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# Distribution of circulating and glomerular IgG subclasses against full-length anti-phospholipase A2 receptor and its epitopes in primary membranous nephropathy

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# DISTRIBUTION OF CIRCULATING AND GLOMERULAR IGG SUBCLASSES AGAINST FULL-LENGTH ANTI- PHOSPHOLIPASE A2 RECEPTOR AND ITS EPITOPES IN PRIMARY MEMBRANOUS NEPHROPATHY

By

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# DISTRIBUTION OF CIRCULATING AND GLOMERULAR IGG SUBCLASSES AGAINST FULL-LENGTH ANTI- PHOSPHOLIPASE A2 RECEPTOR AND ITS EPITOPES IN PRIMARY MEMBRANOUS NEPHROPATHY

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ABSTRACT

**Objective:** Research towards understanding $PLA_2R$, the human antigen of primary membranous nephropathy has steadily gained ground since its discovery in 2009. This autoimmune kidney disease features a unique immunological character of high IgG4 prevalence, both in circulation and as immune complexes deposited in tissue. We seek to characterize and better understand the distribution of all IgG subclasses between serum and glomerular deposits, as well as characterize IgG reactivity directed against both full-length $PLA_2R$ and its immunogenic components.

**Methods:** Using biopsy data obtained from renal pathology centers, we identified 13 patients with primary membranous nephropathy as well as biopsy immunofluorescence data for all IgG subclasses. We compared anti-$PLA_2R$ staining in glomeruli to serum anti-$PLA_2R$ using western blot, and analyzed concordance of subclass distribution between the two sets of data using Cohen's kappa score. We also studied and similarly analyzed, using western blot, subclass distribution of IgG against $PLA_2R$ epitopes CysR, CTLD1, and CTLD4-8.

**Results:** All 13/13 (100%) patient samples were positive for circulating anti-$PLA_2R$ IgG4 in western blot, with 11/13 (84.6%) positive for IgG3, 6/13 (46.2%) for IgG2, and 11/13 (84.6%) for IgG1. When compared with biopsy immunofluorescence these results exhibited fair agreement for IgG4, IgG3, and IgG1; IgG2 was discordant with corresponding biopsies. For reactivity against $PLA_2R$ epitopes, 11/12 (91.7%) samples were positive for anti-CysR IgG4, 5/12 (41.7%) positive for IgG3, 0/11 (0.0%) for IgG2, and 8/11 (72.7%) for IgG1. Reactivity against epitopes CTLD1 and CTLD4-8 was detected less frequently than against CysR, though IgG4 was still the predominant subclass in almost all cases.

**Conclusion:** In general, levels of circulating IgG subclasses directed against $PLA_2R$ is concordant between serum and in biopsy, and as such serum anti-$PLA_2R$ can act as a good proxy for all IgG subclasses found in glomerular deposits. Furthermore, both full-length $PLA_2R$ and its extracellular domain containing the CysR epitope exhibit concordance between IgG3 and IgG4 levels, demonstrating potential for anti-CysR autoantibodies to be a good indicator for primary MN in addition to anti-$PLA_2R$.

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## LIST OF ABBREVIATIONS

ANOVA ..... Analysis of variance

BSA ..... Bovine serum albumin

CTLD ..... C-type lectin domain

CysR ..... Cysteine-rich ricin

ELISA ..... Enzyme-linked immunosorbent assay

FN ..... Fibronectin

IFA ..... Indirect immunofluorescence assay

GBM ..... Glomerular basement membrane

HEK ..... Human embryonic kidney

HGE ..... Human glomerular extract

MAC ..... Membrane attack complex

MN ..... Membranous nephropathy

NEP ..... Neutral endopeptidase/neprilysin

PAGE ..... Polyacrylamide gel electrophoresis

PBS ..... Phosphate buffered saline

$PLA_2R$ ..... M-type phospholipase $A_2$ receptor

SDS ..... Sodium dodecyl sulfate

TBS ..... Tris buffered saline

THSD7A ..... Thrombospondin type-1 domain-containing 7A

UPCR ..... Urine protein-creatinine ratio

# INTRODUCTION

## Overview

Membranous nephropathy (MN) is an autoimmune kidney disease affecting the glomerulus, characterized by *in situ* subepithelial immune complex formations depositing within the glomerular basement membrane (GBM) of podocytes, and subsequently causing damage to glomerular filtration barrier (Salant & Cattran, 2010). It is the most common cause of nephrotic syndrome in adults aged 60 or older, and though more rare in children (Jaipaul, 2013), often leads to severe proteinuria and edema in patients. About one-third to half of all patients experience eventual progression to end-stage renal disease and kidney failure within several years, though spontaneous clinical remission as well as abated disease progression may also occur (UNC Kidney Center, n.d.). The first-year mortality rate for end-stage renal disease from all causes is approximately 200 per 1000 patients, and as such MN represents a notable healthcare concern in the United States (United States Renal Data System, 2014). Clinically about three-quarters of cases (Cattran & Rennke, 2014) are classified as primary MN—previously called idiopathic MN—and have no clear etiology. In contrast, the remaining quarter of cases develop secondary to a pre-existing medical condition or infection, exposure to chemicals and toxins, or as a side-effect of certain drug treatment regimens (Table 1) (Salant & Cattran, 2010).

**Table 1, Causes of secondary membranous nephropathy.** A non-comprehensive list of diseases and conditions associated with and causative of secondary MN *(Salant & Cattran, 2010, p. 240)*.

| Category | Condition |
|---|---|
| Immune disorders | Systemic lupus erythematosus |
| | Graft-versus-host disease |
| | Lupus nephritis |
| Infections | Hepatitis B |
| | Hepatitis C (less common) |
| | HIV |
| Drugs/toxins | Penicillamine |
| | Mercury-containing compounds |
| | NSAIDs, COX-2 inhibitors |
| Other | Malignancies and tumors |
| | Sarcoidosis |
| | Kidney transplantation (de novo MN) |

## A brief history and background of MN

MN was initially defined as nephrotic glomerulonephritis, a condition characterized by edema and severe proteinuria with a more "distinguished pathology" as the disease progressed (Jones, 1957). Subsequently, an inducible model of rat MN was developed and studied extensively as an animal model for the disease in humans, long before identification of an endogenous human antigen (Heymann, et al., 1965). Eponymously named Heymann nephritis, experiments involving this model led to the discovery of the 330-kDa glycoprotein gp330 (eventually to be identified as a component of megalin), a rat podocyte and tubule brush-border protein antigen responsible for the formation of granular immune complex deposits in the glomerulus and development of proteinuria (Kerjaschki & Farquhar, 1982). Thereafter a 400-kDa protein (LRP2) similar to megalin was also found in proximal tubules of human kidneys, though LRP2 is localized to neither the glomerulus nor detectable in immune complexes, and thus

insufficient to explain development of MN in humans (Kerjaschki, et al., 1987). In the Heymann nephritis animal model, podocyte damage and subsequent proteinuria is caused by IgG-mediated complement cascade activation (Kerjaschki & Neale, 1996). Despite the knowledge Heymann nephritis provided towards understanding animal models of MN, a firm connection to the human disease had yet to be made at this point because the human antigenic target still remained elusive. Nevertheless, progress occurred with the identification of the approximately 90-kDa protein neutral endopeptidase (NEP, also neprilysin), a human podocyte antigen that is also expressed in various other tissues, including the fetal placenta. NEP is responsible for a form of fetal MN that develops prenatally (Debiec, et al., 2002). The resultant alloimmune MN is characterized by circulating maternal anti-NEP antibodies which, due to maternal deficiency of a gene coding for NEP and previous sensitization from miscarriage, cross the placenta and specifically target fetal podocytes (Debiec & Ronco, 2007). This discovery represents the first endogenous podocyte antigen directly responsible for MN in humans. And though NEP is not responsible for the primary disease, similarities between alloimmune MN and Heymann nephritis offered more support that a glomerular antigen was also responsible for development of primary MN in humans.

A human antigen specific for primary MN was eventually discovered in 2009, which identified the 185-kDa M-type phospholipase $A_2$ receptor ($PLA_2R$): $PLA_2R$ (Figure 1) is a member of the mannose receptor family and a type-1 transmembrane glycoprotein whose immunogenicity is sensitive to reducing conditions but not N-glycosylation (Beck, et al., 2009). In this study, serum from approximately 70% of

patients with primary MN—and none in healthy controls or those with secondary MN—were found to specifically bind both native and recombinant human $PLA_2R$. Like megalin and NEP, $PLA_2R$ is expressed on the basal surface of podocytes, though its expression is greatly increased in humans compared to other species. Circulating anti-$PLA_2R$ IgG binds to podocytes, becomes capped, and is shed and deposited in the podocyte GBM to form immune complexes, whereupon clinical symptoms such as proteinuria and edema can result by as-of-yet unclear pathological mechanisms (Hofstra & Wetzels, 2012).

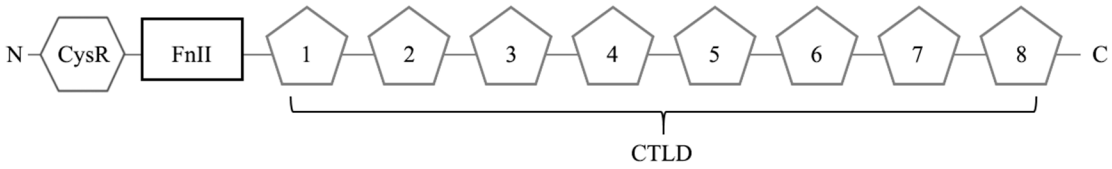



**Figure 1, Extracellular domains of $PLA_2R$.** The N-terminus contains a cysteine-rich ricin (CysR) domain, followed by a collagenous fibronectin-like type II domain (FNII), C-type lectin domains (CTLD) 1-8, and finally a transmembrane and intracellular region towards the C-terminus. The receptor is believed to be capable of folding its extracellular regions via disulfide bonding: its conformation-dependent epitopes are sensitive to reducing conditions, in which reactivity to anti-$PLA_2R$ autoantibodies is largely abolished *(Beck, 2015)*. Factors leading to $PLA_2R$ acting as an autoantigen have yet to be completely determined.

Prior to the discovery of $PLA_2R$, analyzing renal biopsies using light and electron microscopy was the primary method to obtain a definitive MN diagnosis. The histological characteristics and ultrastructure of glomeruli, along with associated clinical features, were first outlined by Jones in 1957. Prominent features visible in light microscopy include glomerular capillary wall and GBM thickening, with subepithelial "spikes" that extend from the GBM around immune complex deposits (Figure 2B & 2C);

the granular deposits and thickened GBM can be visualized in more detail by electron microscopy (Figure 3) (Elsanjak & Prabhakar, 2011). Ultrastructural staging of MN by electron microscopy uses the aforementioned subepithelial spikes to characterize the extent of GBM expansion, with earlier stages associated with more favorable clinical outcomes (Ehrenreich & Chung, 1968).

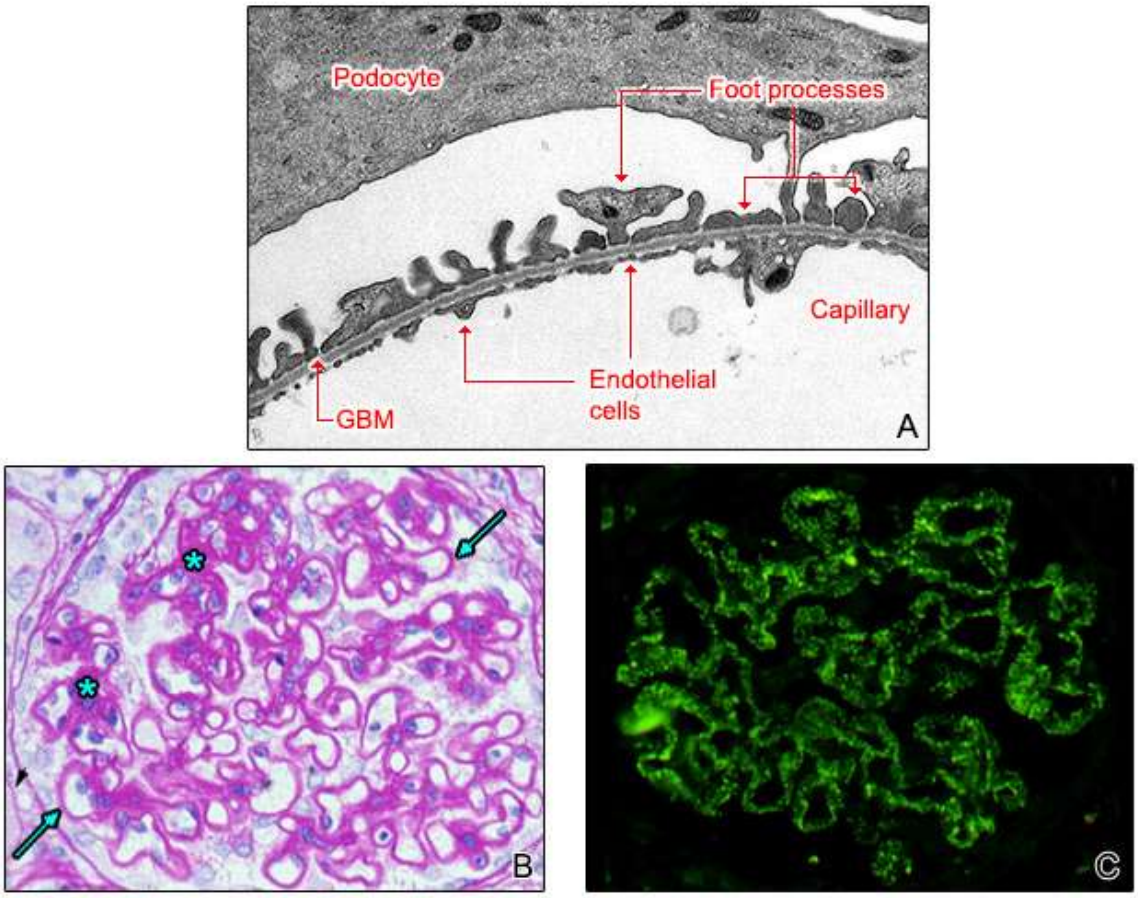



**Figure 2, Histology in normal and membranous glomeruli.** (A) Ultrastructure of normal glomerulus visualized using electron microscopy, showcasing elements of the filtration barrier. (B) Glomerulus with MN, showing GBM thickening (arrows) and mesangial hyperplasia (asterisks). (C) Immunofluorescence of glomerulus with MN, showing granular IgG deposition along capillary walls. Adapted from *(Boston University School of Medicine, n.d.)* and *(Cattran & Rennke, 2014)*.

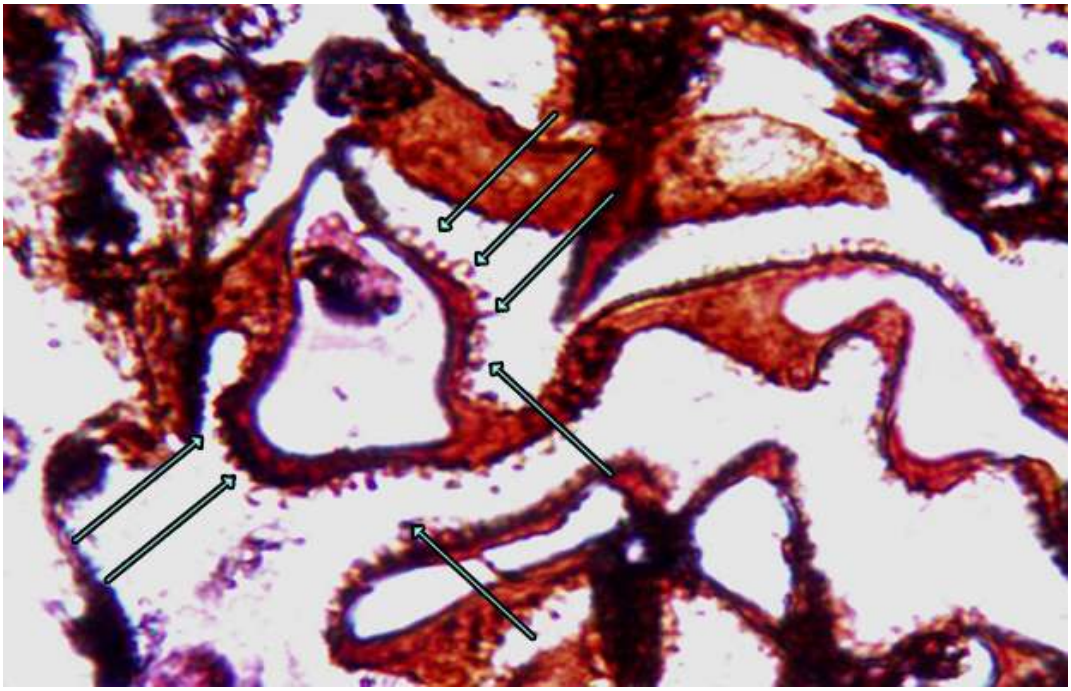


**Figure 3, Subepithelial spikes on membranous glomerulus.** Close-up light microscopy of glomerulus with membranous nephropathy, showcasing characteristic GBM "spikes" (arrows) extending around immune complex deposits. Adapted from *(Elsanjak & Prabhakar, 2011)*.

**Mechanisms and pathology: IgG and the complement cascade**

The proposed mechanism of damage and disease progression requires an overview of immunoglobulins, of which there are five isotypes in humans, and its role in complement activation. First described by von Behring and Kitasato in the late 19$^{th}$ century, subsequent research demonstrated that serum antibodies were able to offer protection against disease by neutralizing toxins as well as lysing bacteria (Kuby, 1997). There are five antibody isotypes which differ in the composition of their heavy chains, providing the molecule with its unique properties. Of these, IgG constitutes the majority isotype in human serum, and is largely responsible for humoral immunity via opsonization, neutralization, and complement activation (Kuby, 1997), as well as almost

exclusively representing the isotype found in immune complex deposits of primary MN (Salant & Cattran, 2010). IgG is composed of four subclasses each with a range of individual properties: susceptibility to proteolytic cleavage, hinge flexibility, efficacy in binding chemically distinct targets are among these (Table 2). More specifically, in primary MN the autoantibodies against $PLA_2R$ are predominantly of the IgG4 variety with lesser contribution from the other subclasses as seen in immune complex deposits (Doi, Mayumi, Kanatsu, Suehiro, & Hamashima, 1984), and also in serum as measured by ELISA and IFA (indirect immunofluorescence assay) (Hofstra, et al., 2012). This is in contrast with the expected distribution of circulating IgG subclasses within healthy adults (Hamilton, 2001), though during earlier stages of primary MN progression the immune deposits contain more IgG1 and IgG3 (Huang, et al., 2013). Owing to these observations, predominance of IgG4 in glomerular deposits frequently becomes a distinguishing feature and diagnostic marker of primary MN, and its majority presence is often contrasted to secondary MN. For example, in cases of membranous lupus nephritis biopsy immunofluorescence indicated a more equal distribution of IgG subclasses in glomerular deposits, with IgG1 and IgG2 more frequently detected in both serum and biopsy instead of IgG4 (Kuroki, et al., 2002). In addition, patients with malignancy-associated secondary MN also exhibited heightened IgG1 and IgG2 deposition in glomeruli, demonstrating a distinguishing feature between primary from secondary forms of MN (Ohtani, et al., 2003).

**Table 2, Chemical and biological properties of IgG.** Key properties of the four IgG subclasses in healthy adults. Of note, circulating IgG4 represents the least common subclass by amount and percentage in humans, and is incapable of activating complement via the classical pathway. Data obtained from *(Meulenbroek & Zeijlemaker , n.d.)* and *(Hamilton, 2001, pp. 28-29)*.

| **Property** | **Subclass** | | | |
|---|---|---|---|---|
| | IgG1 | IgG2 | IgG3 | IgG4 |
| Serum amt. g/L (%) | 8 (60-70) | 4 (14-20) | 0.8 (4-8) | 0.4 (2-6) |
| Molecular mass (kDa) | 146 | 146 | 170 | 146 |
| Hinge region | | | | |
| # of residues | 15 | 12 | 62 | 12 |
| # of disulfide bonds | 2 | 4 | 11 | 2 |
| Immunogenicity | | | | |
| vs. proteins | Moderate | Weak | Moderate | Weak |
| vs. polysaccharides | Weak | Moderate | None | None |
| vs. allergens | Weak | None | None | Moderate |
| Complement activation | | | | |
| C1, classical | Moderate | Weak | Strong | None |
| C3, alternative | None | Weak | None | Weak |

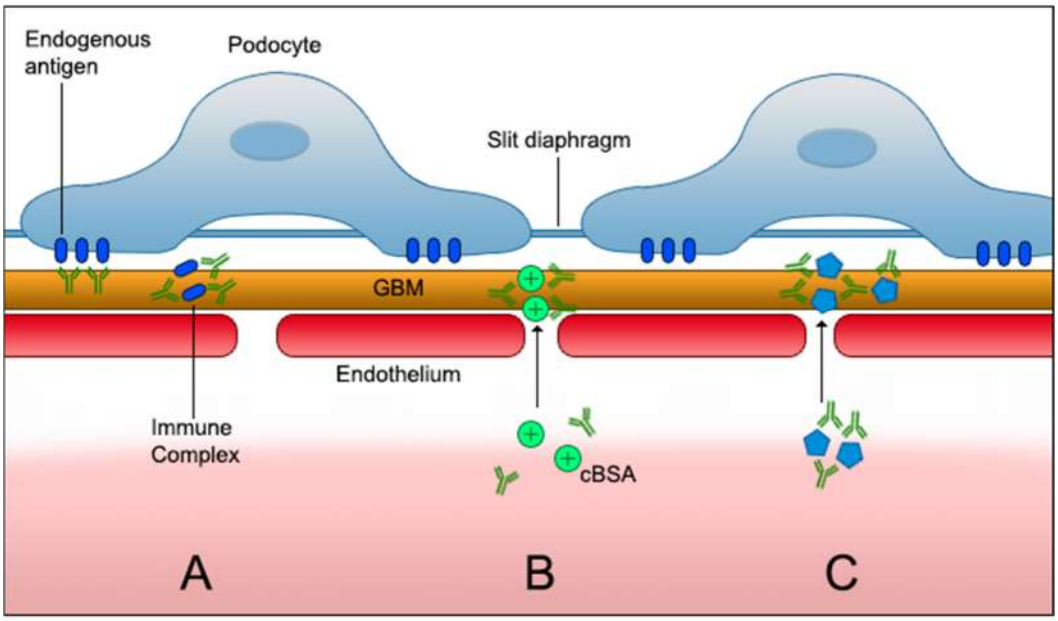



**Figure 4, Formation of subepithelial immune complexes.** Methods for immune complex accumulation in the GBM: (A) circulating antibodies which bind an endogenous antigen (e.g., $PLA_2R$, NEP) on podocyte basal surfaces are eventually shed onto the GBM, forming deposits *in situ* and resulting in damage to the podocyte filtration barrier; (B) positively-charged proteins (e.g., cBSA) are attracted to the negatively-charged GBM, becoming an exogenous antigen; (C) pre-formed circulating immune complexes may cross into and deposit on the GBM. Adapted from *(Beck & Salant, 2014)*.

There are several mechanisms that can cause immune complexes to form on the podocyte GBM (Figure 4). In addition to an *in situ* autoimmune process (such as in the cases of NEP and $PLA_2R$), both preformed circulating immune complexes and cationic bovine serum albumin (cBSA) can lead to buildup of glomerular deposits, though these do not contribute to the pathogenesis of primary MN (Beck & Salant, 2014). In animal models and rare cases of childhood MN (Debiec, et al., 2011), cBSA was able to cross the filtration barrier and become embedded in the anionic GBM, thus becoming a target for circulating anti-BSA antibodies (Border, Ward, Kamil, & Cohen, 1982). In secondary MN, causative diseases such as systemic lupus erythematosus initially involves immune complex formation in circulation before depositing in the GBM (Mok & Lau, 2003). Once immune complexes have accumulated, it was believed that in models of passive Heymann nephritis the IgG contained in these deposits caused podocyte injury and proteinuria through activation of the complement cascade, evidenced by concurrent presence of complement component C3 (Salant, Belok, Madaio, & Couser, 1980). To introduce complement system briefly (Figure 5), a series of serum proteins beginning with component C1 in the classical pathway—and cleavage product C3b in the alternative pathway—is enzymatically cleaved, each reaction product catalyzing the next, eventually activating C3 convertase and forming the membrane attack complex (MAC) composed of complement proteins C5b-C9 (Kuby, 1997). The MAC inserts into the target cell's lipid bilayer and forms transmembrane channels, promotes a local cytotoxic environment rich in oxidative species and inflammatory agents (Kerjaschki & Neale, 1996), and leads to "disruption of the actin cytoskeleton [and] causes altered cell-matrix

adhesion and loss or displacement of slit diaphragms…and loss of the filtration barrier to protein" (Salant & Cattran, 2010). Further research suggested that complement activation in human MN underwent a parallel mechanism, in which for most cases presence of C3 and its breakdown products can be detected (Segawa, et al., 2010).

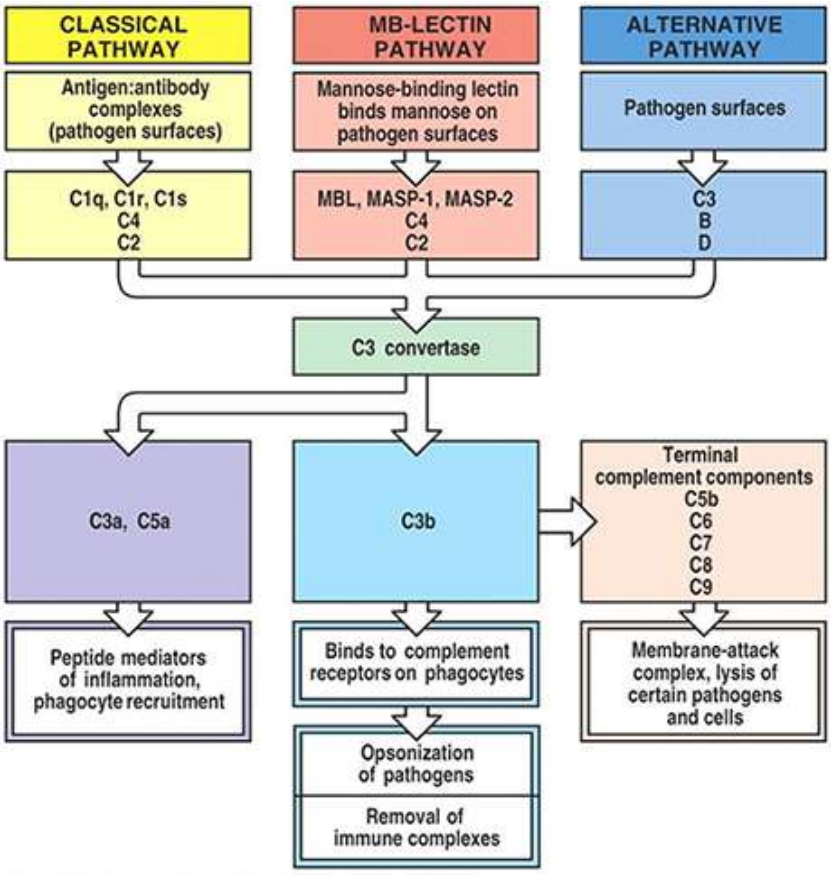



**Figure 5, Roles and activation of the complement system.** Three pathways leading to activation of the complement cascade. The classical pathway is involved in secondary MN, and initiated by C1 interaction with antibody-antigen complexes. All three pathways leads to C3 convertase and the formation of C5b-9 (MAC), a molecule which promotes a cytotoxic environment by way of reactive oxygen species and inflammatory agents. Cleavage products C3a, C5a, and C3b have additional roles in the innate immune system. Taken from *(Janeway, Travers, Walport, & Shlomchik, 2004)*.

While pathogenesis due to complement activation (as in rat the Heymann nephritis model) is a tempting culprit, there are still unanswered questions regarding the disease mechanism in humans: the glomerular immune complex deposits of primary MN contains predominantly IgG4, which is incapable of fixing complement components via the classical pathway as previously described (Meulenbroek & Zeijlemaker , n.d.). This is in contrast to the increased amounts of other IgG subclasses found in glomerular deposits of patients with secondary MN, which provides evidence for a clearer pathological mechanism. Nevertheless, it has been proposed that the relatively larger IgG1 and IgG3 deposition initially present in early phases of primary MN may be sufficient to induce complement-mediated podocyte injury (Huang, et al., 2013). Occasional but sparse detection of C3 in biopsies of individuals specifically with primary MN lends some credence to involvement of the classical pathway, though currently there is not enough conclusive data to confirm this hypothesis (Ma, Sandor, & Beck, 2013).

**Applications of research and clinical connections**

The literature in this field has been rapidly growing, and reveals new questions in addition to answering old ones. Firstly, as IgG4 is unable to fix complement its predominance in primary MN calls into question its pathogenicity, and attempts to test this by overexpressing $PLA_2R$ in animal models have been unsuccessful (Ronco & Debiec, 2014), though there is some circumstantial evidence implicating its involvement in complement cascade activation via the mannose-binding lectin pathway (Ma, Sandor, & Beck, 2013). Secondly, in the pivotal study circulating IgG against $PLA_2R$ was

successfully detected in 70% of patients with biopsy-proven primary MN (Beck, et al., 2009), though this percentage varied considerably from approximately half positive up to 86% (Hofstra & Wetzels, 2012). Serum anti-$PLA_2R$ autoantibodies have been shown to exhibit very high specificity, in contrast to their comparatively lower sensitivity—this observation may be attributed to several factors, from differing experimental methods to immunological reasons concerning each patient. For example, a cohort of Japanese nephrotic patients exhibited lower overall anti-$PLA_2R$ titers compared to other Asian and European cohorts (Akiyama, et al., 2014), suggesting that immunological aspects MN may manifest differently among distinct ethnic groups. Additionally, MN has been shown to undergo immunological remission and relapse (Figure 6) in which lowered serum anti-$PLA_2R$ levels are associated with higher likelihood of spontaneous clinical remission (Hofstra, et al., 2012). The clinical disease can persist for several months after immunological remission occurs (Beck & Salant, 2014), making it difficult to pinpoint an exact place along the disease continuum. This time lapse and the uncertainty in measurement it brings can contribute to observed interstudy variations in sensitivity. Therefore, it is possible that due to methodological and population differences among the various studies, including elapsed time between dates of patient biopsy, their corresponding date of data collection, and time-delayed effects of therapeutic regimens, heterogeneous results may be produced when assessing diagnostic sensitivity of anti-$PLA_2R$ (Du, et al., 2014).

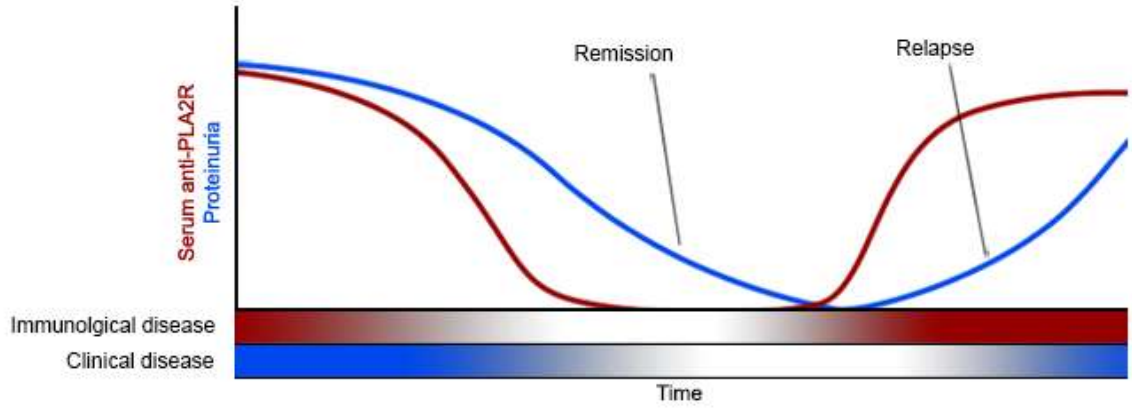



**Figure 6, Time course of serum anti-PLA$_2$R and clinical symptoms.** Hypothetical case of primary MN conceptually illustrating the level of serum anti-PLA$_2$R (immunological disease) and level of proteinuria (clinical disease). As with all typical cases, there is a delay between the onset of immunological remission and clinical remission, which can be on the order of several months to upwards of a year.

With some rare exceptions, anti-PLA$_2$R autoantibodies are a risk factor for developing nephrotic proteinuria, a common symptom of primary MN (Hoxha, Harendza, Pinnschmidt, Panzer, & Stahl, 2014). The exact pathogenic nature of anti-PLA$_2$R is still unknown, though this does not necessarily diminish the clinical value of measuring these autoantibodies: studies have shown that serum anti-PLA$_2$R levels correlate with urinary protein excretion, are typically absent during clinical remission, and predict clinical relapse and long-term outcome of primary MN patients (Hofstra, Beck, Beck, Wetzels, & Salant, 2011). Their relationship is more definitively demonstrated with depletion of circulating anti-PLA$_2$R autoantibodies induced by rituximab—a drug used for immunosuppression therapy in primary MN—whereby clinical remission is more likely after anti-PLA$_2$R levels subside (Beck, et al., 2011). Using a variety of different conditions and methodologies measuring both serum and glomerular autoantibodies,

researchers were able to demonstrate the diagnostic and prognostic value of anti-$PLA_2R$: patients with greatly reduced autoantibody levels were much more likely to eventually undergo clinical remission compared to those with persistent levels within a similar time frame. In our study, we also hope to examine further this link between deposited glomerular IgG and corresponding anti-$PLA_2R$ IgG subclasses in circulation.

A developing topic of current primary MN research is characterizing epitope specificity of anti-$PLA_2R$ autoantibodies. It is widely known that $PLA_2R$ exists in both linear and folded configurations, and contains conformational epitopes. In addition to our own work, two research groups have recently published their findings identifying an immunodominant region of $PLA_2R$. A research group from UCLA has created a panel of $PLA_2R$ constructs, beginning with the CysR-FNII epitopes and for each new construct adding subsequent CTLD epitopes toward the C-terminus, ultimately assembling the entire extracellular $PLA_2R$ domain (Kao, Lam, Waldman, Glassock, & Zhu, 2015). They conclude that the immunodominant epitope lies exclusively within the CysR-FNII-CTLD1 domains, as its immunogenicity and susceptibility to reducing conditions mirror that of full-length $PLA_2R$. Another research group from the United Kingdom furthered these findings by demonstrating immunogenicity of a distinct 31-chain polypeptide within the CysR domain (Fresquet, et al., 2015). While both groups also acknowledged occasional autoantibody reactivity to the CTLD4-8 regions, the nature of this interaction is more surreptitious with regards to primary MN, and its detection is generally inconsistent. The preliminary work done by these two groups explores the well-established paradigm of IgG4 predominance in primary MN, and whether this also

extends to $PLA_2R$ epitopes or not. In this study, and in addition to reactivity against full-length $PLA_2R$, we hope to build upon knowledge regarding reactivity of circulating IgG subclasses against epitopes within $PLA_2R$.

**Specific aims of research**

Though IgG4 predominance has been well-established for primary MN by both serology and biopsy, our study seeks to explore in greater detail the relationship between all IgG subtypes, whether in circulation or deposited in glomeruli.

In particular, we used data in biopsy reports of patients with primary MN, obtained from renal centers which routinely perform biopsy immunofluorescence for the four IgG subclasses. The biopsy data will then be compared to corresponding serum samples collected from these same patients, and the concordance between strength of IgG subclasses in serum and in biopsy will be examined. We will also analyze the extent to which IgG subclass reactivity against $PLA_2R$ epitopes agree with reactivity against the full-length molecule. Finally, we will seek to find any correlations between subclass responses and baseline clinical characteristics, as provided for by the biopsy reports, for each patient.

Characterization of anti-$PLA_2R$ IgG responses will ideally add to the growing field of MN research. We hope that these results will further reinforce the relationship between circulating and deposited autoantibodies against full-length $PLA_2R$ and its epitopes for the IgG subclasses.

# METHODS

## Samples and selection

In our experiments we targeted $PLA_2R$ contained in human glomerular extract (HGE). To prepare HGE we obtained healthy kidneys that were provided by the New England Organ Bank from deceased individuals. We thawed and cut frozen kidney tissue into a fine mince, which was then processed by sieving (Fisher Scientific Nos. 140, 80, 200) and thorough washing with cold PBS. The remaining glomeruli we homogenized and proteins were extracted with SDS. Finally, we depleted any contaminating human IgG using Immobilized Protein G Plus (Fisher Scientific), and verified depletion of residual IgG using western blot (data not shown). The processed HGE aliquots were then prepared under non-reducing conditions for use in SDS-PAGE, and heated at 95$^{o}$C for 5 minutes. Thus, HGE proteins used in subsequent western blots were free of most hydrogen bonds and non-polar hydrophobic interactions while still maintaining the disulfide bonds necessary for reactivity with anti-$PLA_2R$ (Ophardt, 2003).

Biopsy reports with clinical characteristics and corresponding frozen patient serum samples were obtained from renal pathology centers which routinely perform IgG immunofluorescence (Table 3). These staining procedures visualized all IgG subclasses deposited in glomeruli, and may include other IgG in addition to those specific for $PLA_2R$. We selected those patients who were diagnosed for primary MN and seropositive for anti-$PLA_2R$. We further refined our selection by only choosing those patients with data on immunofluorescence staining for all IgG subclasses, and our criteria yielded a

final selection of 13 patient samples (Figure 7). A full table of all patients and their associated clinical information is also available in the appendix (Table 16)

**Table 3, Summary data for patient samples.** Patient data was obtained from biopsy reports. [1]Circulating IgG of all subclasses against human recombinant $PLA_2R$ as measured by ELISA. [2]Immunofluorsecence scores transcribed directly from biopsy reports. [4]Months elapsed after biopsy date that serum sample was taken. Values are reported as mean ± standard deviation or *n* (%).

| **Patient data, n = 13** | | |
|---|---|---|
| | Mean ± S.D. | Range |
| Age (years) | 60.3 ± 14.2 | 34, 79 |
| Male, *n* | 7 (53.8) | |
| Ethnicity, *n* | | |
| Asian | 2 (15.4) | |
| Caucasian | 10 (76.9) | |
| Clinical information | | |
| Serum creatinine (mg/dL) | 1.60 ± 0.77 | 0.67, 3.02 |
| Serum albumin (g/dL) | 2.70 ± 0.80 | 1.7, 4.0 |
| UPCR | 9.78 ± 5.43 | 3.13, 19.70 |
| Anti-$PLA_2R$[1] (RU/mL) | 150.5 ± 152.2 | 13.7, 493.4 |
| Immunohistological staining[2] | | |
| C3 | 2.0 ± 1.0 | 0, 3 |
| C1q | 0.5 ± 0.7 | 0, 2 |
| Total IgG | 3.7 ± 0.4 | 3, 4 |
| Months after Bx[3] | 5.2 ± 5.7 | -0.6, 19 |

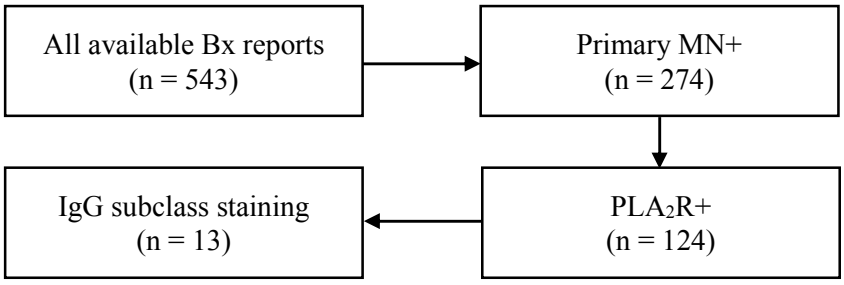



**Figure 7, Selection criteria for patient samples.** The full list of 543 patients, from which we refined our final selection of patient samples. Diagnosis of primary MN had been previously determined by biopsy.

**Western blot: full-length $PLA_2R$ and $PLA_2R$ epitopes**

We followed standard procedures for western blot with HGE, under non-reducing conditions with SDS-PAGE and nitrocellulose membrane for transfer electrophoresis. Primary antibody consisted of patient serum samples diluted to 1:100 (all serum and antibodies were diluted in 1x TBS/0.2% Tween-20 with 10% nonfat dry milk). For the secondary antibodies, we used affinity-purified sheep anti-human IgG1-4 (Table 4) at dilutions recommended by the manufacturer (Binding Site). For our detection antibodies we used donkey anti-sheep IgG conjugated with horseradish peroxidase (Sigma) diluted to 1:10,000 and detected by chemical luminescence.

**Table 4, HRP-conjugated secondary antibody.** All sheep anti-human antibodies were diluted in 1x TBS/0.2% Tween-20 with 10% nonfat dry milk. Dilutions were recommended by the manufacturer (Binding Site).

| Subclass | Dilution |
|---|---|
| Anti-IgG1 | 1:4000 |
| Anti-IgG2 | 1:4000 |
| Anti-IgG3 | 1:2500 |
| Anti-IgG4 | 1:3000 |

In addition to HGE, we also performed western blots for constructs of $PLA_2R$ epitopes. Dr. Dana Sandor and Dr. Hong Ma cultured and transfected human embryonic kidney (HEK) 293T cells with vectors containing CysR, CTLD1, and CTLD4-8 (see Figure 1 for reference), which we used to asses patient serum reactivity to individual $PLA_2R$ epitopes. In some cases, the serum dilutions used for western blot was too low to detect PLA2R epitopes, and as such we used higher serum concentrations accordingly. Experiments pertaining to patient serum samples MN-01 and MN-10 were performed

using 1:100 dilution; samples MN-02 and MN-03 using 1:25 dilution; samples MN-04 through MN-09, MN-11, and MN-13 using 1:50 dilution.

**Statistical analysis**

Where appropriate, data for descriptive statistics were presented as mean ± S.D, as there were no outliers present for these summaries. Western blots were assessed semi-quantitatively and assigned scores ranging from 0 (no response) to 4+ (very strong response). For western blot and biopsy immunofluorescence scores, IgG subclass predominance was established using the Mann-Whitney *U* test. We also compared patient clinical characteristics and western blot results, and analyzed correlation between these using Kendall rank correlation coefficient. Additionally, western blot scores were also assigned to one of three distinct strength categories (Table 5). With these values, we measured concordance between scores from biopsy immunofluorescence and western blot using Cohen's kappa. For all statistical analysis we used MedCalc v12.5 (MedCalc Software) and StataMP 13 (StataCorp). A critical value of $P \leq 0.05$ was considered significant.

**Table 5, Ordinal assignment of western blot and biopsy scores.** Western blot scores were graded based on intensity of bands, and biopsy scores for immunofluorescence were transcribed as provided in the biopsy reports. These numerical scores were then assigned to one of three strength categories and used for concordance analysis.

| Category | Scores | |
|---|---|---|
| | Western blot | Biopsy |
| Negative | 0 | 0, 0.5+ |
| Weak | 1+, 2+ | 1+ to 2.5+ |
| Strong | 3+, 4+ | 3+ to 4+ |

# RESULTS

## Anti-PLA$_2$R IgG subclasses in western blot and biopsy

All serum samples were from patients with primary MN, confirmed by biopsy staining and clinical criteria. The results for western blot (Figure 10) were scored using criteria as previously described, and a detailed overview of scores for each sample is provided (Tables 6 & 7). We observed 13/13 (100%) samples to have IgG4 autoantibodies reactive with native PLA$_2$R in HGE. Other subclasses included 11/13 (84.6%) IgG1, 6/13 (46.2%) IgG2, and 11/13 (84.6%) IgG3. When the band on western blot for a subclass was clearly the strongest relative to all others, we deemed that subclass to be predominant (Figure 8). If instead two or more of the strongest subclasses had similarly intense bands on western blot, we deemed those subclasses to be co-dominant. Following this criteria, 10/13 (76.9%) samples were IgG4 predominant, 1/13 (7.7%) samples were IgG3 predominant; 2/13 (15.4%) samples were IgG4 co-dominant with IgG3. As a whole, a semi-quantitative analysis of western blots showed IgG4 scored significantly higher ($P \leq 0.01$) than the other subclasses (Figure 9A).

The same summary and analysis was performed for corresponding biopsy immunofluorescence staining data (Tables 8 & 9). All 13/13 (100%) biopsy samples stained positive for IgG4, 11/13 (84.6%) for IgG3, 10/13 (76.9%) for IgG2, and 12/13 (92.3%) for IgG1. IgG4 was the predominant subclass in 11/13 (84.6%) biopsies and co-dominant with IgG3 in 2/13 biopsies (15.4%). The staining scores indicate strengths of IgG subclass immunofluorescence microscopy, which are transcribed directly from the

biopsy report, and show predominant IgG4 deposition with moderate contribution from the other subclasses (Figure 11). Similar to western blot, IgG4 in biopsy was also scored significantly higher ($P \leq 0.01$) than were the other subclasses (Figure 9B).

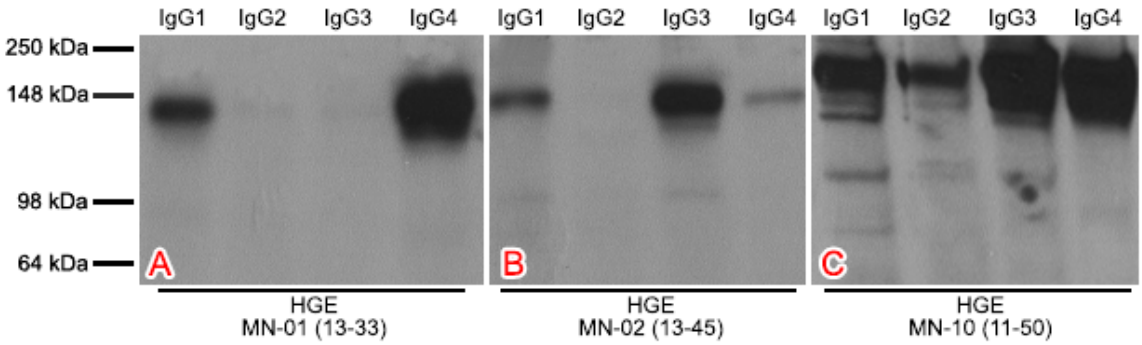



**Figure 8, Human IgG subclass response against HGE.** In western blot, HGE was probed with MN-positive serum (1:100 dilution) and recognized bands approximately corresponding to the size of $PLA_2R$ (~185 kDa), though actual sizes may vary due to extent of N-glycosylation. As examples, shown here are: (A) IgG4 predominance; (B) IgG3 predominance; (C) IgG3 and IgG4 co-dominance.

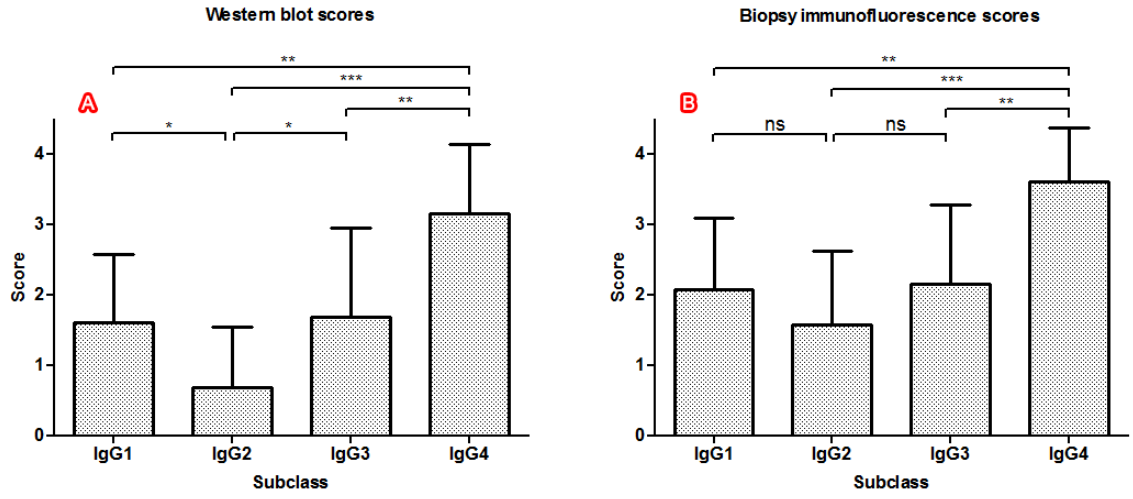



**Figure 9, IgG subclass predominance in western blot and biopsy.** Mean scores with S.D. for A) western blot and B) biopsy immunofluorescence staining. Scoring criteria evaluates strength of bands as follows: 0 indicates negative response, 1+ very weak response, 2+ moderate response, 3+ strong response, 4+ extremely strong response. As the predominant subclass, IgG4 was scored significantly higher compared to all other subclasses. Significance is indicated at $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), or as not significant (ns).

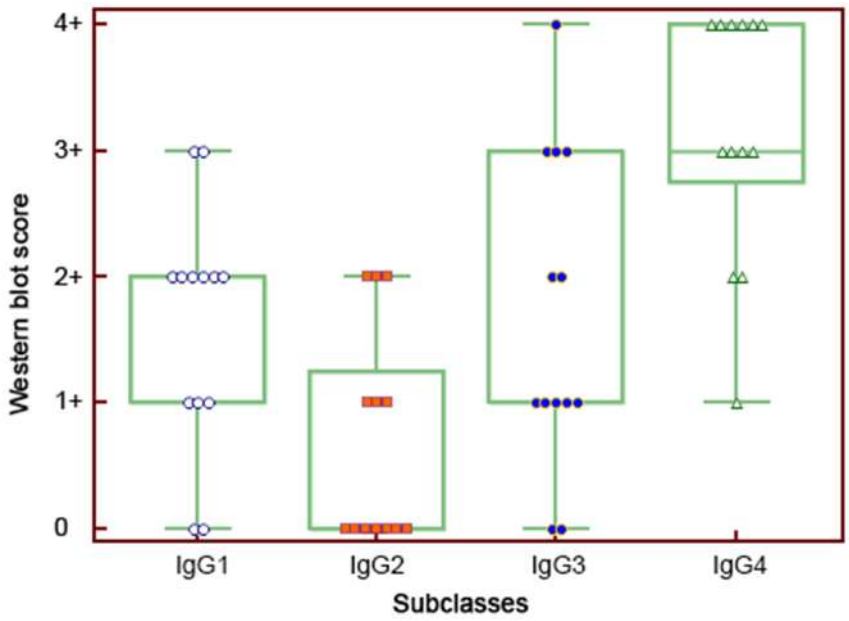



**Figure 10, Western blot IgG subclass scores.** Semi-quantitative results indicating strength of reactivity against $PLA_2R$. Scores range from 0 (no response) to 4+ (very strong response). The predominant subclass in primary MN is IgG4, as is reflected in the western blot results.

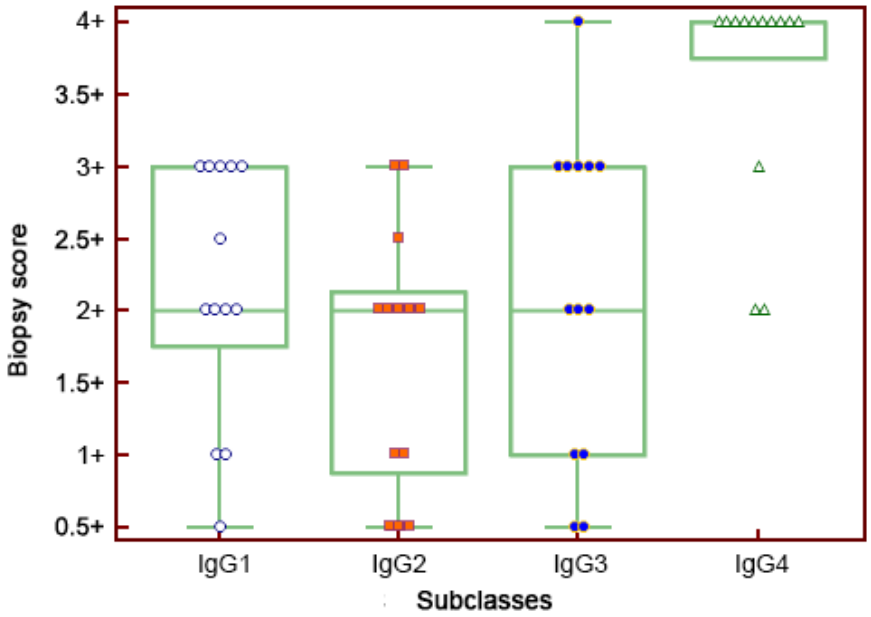



**Figure 11, Biopsy IgG subclass scores.** Strength of immunofluorescence staining for podocyte $PLA_2R$. Scores range from 0 (no staining) to 4+ (very strong staining). Though IgG4 is still the predominant subclass, other subclasses can be more readily found. In general, anti-$PLA_2R$ IgG can be more readily detected in glomeruli than in serum.

**Table 6, Scores for IgG subclasses against HGE.** Semi-quantitative western blot results for patient serum reactivity against $PLA_2R$ in HGE. Scores range from 0 (no response) to 4+ (very strong response). Predominant subclasses are bolded, co-dominant are underlined. Totals reported as *n* (%).

| Subclass response per sample, western blot | | | | |
|---|---|---|---|---|
| | IgG1 | IgG2 | IgG3 | IgG4 |
| MN-01 | 2+ | 0 | 0 | **4+** |
| MN-02 | 2+ | 0 | **3+** | 1+ |
| MN-03 | 2+ | 2+ | 3+ | **4+** |
| MN-04 | 2+ | 0 | 2+ | **3+** |
| MN-05 | 3+ | 0 | 3+ | **4+** |
| MN-06 | 1+ | 2+ | 1+ | **4+** |
| MN-07 | 1+ | 0 | 1+ | **2+** |
| MN-08 | 2+ | 1+ | 1+ | **4+** |
| MN-09 | 1+ | 1+ | 1+ | **3+** |
| MN-10 | 3+ | 2+ | <u>4+</u> | <u>4+</u> |
| MN-11 | 2+ | 1+ | 1+ | **3+** |
| MN-12 | 0 | 0 | 0 | **3+** |
| MN-13 | 0 | 0 | <u>2+</u> | <u>2+</u> |
| Predominant | 0 (0) | 0 (0) | 1 (7.7) | 10 (76.9) |
| Total positive | 11 (84.6) | 6 (46.2) | 11 (84.6) | 13 (100) |

**Table 7, Strength of IgG subclasses against HGE.** Summary of western blot scores for patient serum reactivity against $PLA_2R$ in HGE. Scores are categorized using criteria previously outlined in Table 6. Values reported as *n* (%).

| Subclass response overall, western blot | | | | |
|---|---|---|---|---|
| | IgG1 | IgG2 | IgG3 | IgG4 |
| Negative | 2 (15.4) | 7 (53.8) | 2 (15.4) | 0 (0) |
| Weak | 9 (69.2) | 6 (46.2) | 7 (53.8) | 3 (23.1) |
| Strong | 2 (15.4) | 0 (0) | 4 (30.8) | 10 (76.9) |

**Table 8, Scores for IgG subclass biopsy staining.** Immunofluorescence data collected as-is from routine biopsy reports. Scores range from 0 (no staining) to 4+ (very strong staining). A score of 0.5+ indicates trace amounts of IgG staining, interpreted as negative for statistical analysis. Predominant subclasses are bolded, co-dominant are underlined. Scoring criteria corresponds to relative strength of each subclass via immunofluorescence. Totals reported as *n* (%).

| Subclass response per sample, biopsy | | | | |
|---|---|---|---|---|
| | IgG1 | IgG2 | IgG3 | IgG4 |
| MN-01 | 2+ | 3+ | 1+ | **4+** |
| MN-02 | 3+ | 1+ | 3+ | **4+** |
| MN-03 | 2.5+ | 2.5+ | 3+ | **4+** |
| MN-04 | 2+ | 2+ | 4+ | 4+ |
| MN-05 | 3+ | 2+ | 3+ | **4+** |
| MN-06 | 2+ | 2+ | 1+ | **4+** |
| MN-07 | 3+ | 3+ | 3+ | **4+** |
| MN-08 | 1+ | 0.5+ | 0.5+ | **2+** |
| MN-09 | 3+ | 2+ | 2+ | **4+** |
| MN-10 | 1+ | 0.5+ | 3+ | 3+ |
| MN-11 | 3+ | 1+ | 2+ | **4+** |
| MN-12 | 2+ | 2+ | 2+ | **4+** |
| MN-13 | 0.5+ | 0.5+ | 0.5+ | **2+** |
| Predominant | 0 (0) | 0 (0) | 0 (0) | 11 (84.6) |
| Total positive | 12 (92.3) | 10 (76.9) | 11 (84.6) | 13 (100) |

**Table 9, Strength of IgG subclass biopsy staining.** Summary of glomerular staining for IgG from routine biopsy reports, with trace amounts of staining being interpreted as negative. Scores are categorized using criteria as previously outlined in Table 6. Values reported as *n* (%).

| Subclass response overall, biopsy | | | | |
|---|---|---|---|---|
| | IgG1 | IgG2 | IgG3 | IgG4 |
| Negative | 1 (7.7) | 3 (23.1) | 2 (15.4) | 0 (0) |
| Weak | 7 (53.8) | 8 (61.5) | 5 (38.5) | 2 (15.4) |
| Strong | 5 (38.5) | 2 (15.4) | 6 (46.2) | 11 (84.6) |

**Correlation between anti-PLA$_2$R IgG and clinical characteristics**

Using results from western blot measuring serum anti-PLA$_2$R, we examined correlation between IgG subclasses and clinical characteristics using the Kendall rank correlation coefficient (Table 10). For IgG1, we found significant correlation with Caucasian ethnicity ($\tau$ = -0.47, $P$ = 0.02); for IgG2 with UPCR ($\tau$ = 0.46, $P$ = 0.03); for IgG3 with Caucasian ethnicity ($\tau$ = -0.42, $P$ = 0.04), serum albumin ($\tau$ = -0.51, $P$ = 0.03), and negatively with C3 ($\tau$ = -0.40, $P$ = 0.04); for IgG4 with serum anti-PLA$_2$R IgG ($\tau$ = 0.48, $P$ = 0.02) and negatively with C1q ($\tau$ = -0.53, $P$ = 0.01).

**Table 10, Correlation between clinical data and IgG subclasses.** We analyzed significant correlation between anti-PLA$_2$R IgG subclass responses and patient clinical characteristics. For analysis, Caucasian ethnicity = 0, and Asian = 1; for sex, male = 0 and female = 1. Bolded values indicates significance at $P \leq 0.05$.

| **Kendall rank correlation coefficient (τ)** | | | | |
|---|---|---|---|---|
| | IgG1 | IgG2 | IgG3 | IgG4 |
| Age | 0.03 | -0.35 | 0.10 | -0.20 |
| Sex[1] | -0.12 | 0.35 | 0.12 | 0.12 |
| Ethnicity[2] | **-0.47** | -0.03 | **-0.42** | -0.13 |
| Clinical information | | | | |
| Serum creatinine | -0.15 | -0.08 | -0.27 | -0.24 |
| Serum albumin | 0.04 | 0.07 | **0.51** | -0.20 |
| UPCR | 0.21 | **0.46** | 0.19 | 0.09 |
| Anti-PLA$_2$R | 0.39 | 0.14 | 0.21 | **0.48** |
| Immunohistological staining | | | | |
| C3 | -0.07 | -0.02 | **-0.40** | 0.00 |
| C1q | -0.05 | -0.11 | 0.15 | **-0.53** |
| Total IgG | -0.19 | -0.25 | 0.04 | -0.08 |
| Months after Bx | 0.14 | 0.19 | -0.20 | 0.32 |

**Agreement between anti-PLA$_2$R IgG in western blot and biopsy**

We assessed whether the relative amount of IgG found in immune complex deposits, as reflected by the biopsy scores, were concordant or discordant with the amount of serum anti-PLA$_2$R IgG as indicated by western blot (Table 11). We used Cohen's kappa to measure concordance, from a score of 0 (total disagreement) up to a score of 1 (total agreement). A negative kappa indicates that the observed concordance is even less than what would be expected due to random chance. It is also weighted to reflect the fact that a score of 2+ versus 3+ is a greater amount of concordance than a score of 2+ versus 4+. The scale used for interpreting kappa is typically as follows: < 0, less agreement than expected due to chance; 0.01 to 0.20, slight agreement; 0.21 to 0.40, fair agreement; 0.41 to 0.60, moderate agreement; 0.61 to 0.80, substantial agreement; 0.81 to 0.99 almost perfect agreement (Viera & Garrett, 2005). Subclasses IgG1 ($\kappa$ = 0.264), IgG3 ($\kappa$ = 0.381), and IgG4 ($\kappa$ = 0.264) all exhibited fair agreement between biopsy immunofluorescence and western blot. IgG2 ($\kappa$ = -0.140) was the only subclass that exhibited more disagreement than was expected due to chance.

**Table 11, Concordance rating between western blot and biopsy.** We determined the degree of concordance between scores obtained from western blot and biopsy staining for each IgG subclass. Results exhibiting fair agreement or more ($\kappa \geq 0.21$) are bolded. For statistical analysis we used scoring criteria as outlined in Table 6.

| Weighted Cohen's kappa (κ) | | |
|---|---|---|
| | Kappa | Std. error |
| IgG1 | **0.264** | 0.226 |
| IgG2 | -0.140 | 0.174 |
| IgG3 | **0.381** | 0.155 |
| IgG4 | **0.264** | 0.313 |

**IgG subclasses against $PLA_2R$ epitopes in western blot**

We additionally performed western blot using constructs of three individual regions of $PLA_2R$ known to contain distinct epitopes CysR, CTLD1, and CTLD4-8 (see Figure 1 for reference), which we probed with patient serum samples for reactivity against all anti-$PLA_2R$ IgG subclasses (Figure 12). In total, we obtained data for reactive IgG subclasses using serum samples MN-01 through MN-11 and MN-13 (Table 13). MN-07 was the only sample which contained no reactive IgG subclass against any $PLA_2R$ epitope. For each sample, we have also provided a summary of western blot scores, interpreted and assigned as strength of reactivity (Table 12).

The majority of our samples contained anti-$PLA_2R$ IgG directed against epitopes within CysR, the most commonly recognized $PLA_2R$ domain: 11/12 (91.7%) samples contained reactive IgG of any subclass; CTLD1 was positive in 5/12 (41.7%) samples tested; CTLD4-8 was positive in 6/12 (50.0%) samples tested. When examining individual IgG subclasses, positive reactivity from IgG4 was detected against CysR in 11/12 (91.7%) cases, and against both CTLD1 and CTLD4-8 in 5/12 (41.7%) cases. IgG1 reactive against CysR was detected in 8/11 (72.7%) cases, but no IgG1 reactivity was detected against the other two domains. IgG3 reactive against CysR was detected in 5/12 (41.7%) cases; against CTLD1 in 1/11 (8.3%) case; and against CTLD4-8 in 4/12 (33.3%) cases. No reactivity from IgG2 was detected against any epitope within the three $PLA_2R$ constructs. In summary, the majority of positive samples contained anti-$PLA_2R$ reactive against CysR, of which IgG4 was predominant with occasional reactivity from IgG1 and IgG3.

**Table 12, Strength of IgG subclasses against $PLA_2R$ epitopes.** Summary of western blot scores for IgG subclass reactivity against $PLA_2R$ epitopes, each categorized by strength based on previously stated criteria (Table 5). Strong respondents are bolded. Values reported as *n* (% of instances tested for that dataset).

| Subclass response overall, western blot | | | | |
|---|---|---|---|---|
| | IgG1 | IgG2 | IgG3 | IgG4 |
| CysR | | | | |
| Negative | 3 (27.3) | 11 (100) | 7 (58.3) | 1 (8.3) |
| Weak | 3 (27.3) | 0 (0) | 1 (8.3) | 4 (33.3) |
| Strong | **5 (45.5)** | 0 (0) | **4 (33.3)** | **7 (58.3)** |
| CTLD1 | | | | |
| Negative | 11 (100) | 11 (100) | 11 (91.7) | 7 (58.3) |
| Weak | 0 (0) | 0 (0) | 1 (8.3) | 3 (25.0) |
| Strong | 0 (0) | 0 (0) | 0 (0) | **2 (16.7)** |
| CTLD4-8 | | | | |
| Negative | 11 (100) | 11 (100) | 8 (66.7) | 7 (58.3) |
| Weak | 0 (0) | 0 (0) | 4 (33.3) | 3 (25.0) |
| Strong | 0 (0) | 0 (0) | 0 (0) | **2 (16.7)** |

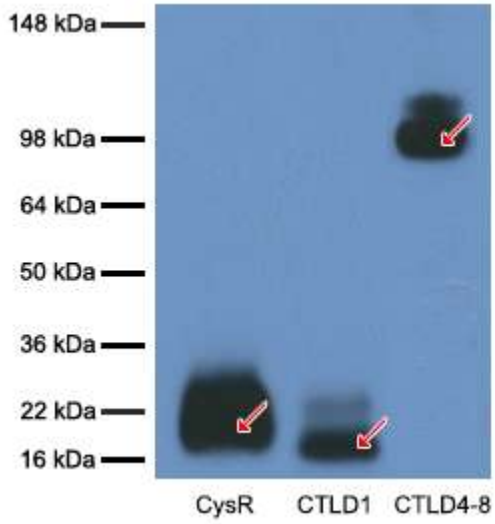



**Figure 12, IgG4 against $PLA_2R$ epitopes in sample MN-01.** Circulating IgG4 (1:100 dilution) reactive against $PLA_2R$ epitopes. Red arrows indicate approximate location of epitopes: ~20 kDa for both CysR and CTLD1, ~90 kDa for CTLD4-8. Expected protein sizes were determined using ProtPram (Swiss Institute of Bioinformatics) by the lab of Dr. Laurence Beck, though actual sizes in assay may vary due to extent of N-glycosylation.

**Table 13, Scores for IgG subclasses against $PLA_2R$ epitopes.** Semi-quantitative western blot results for patient serum reactivity against $PLA_2R$ epitopes. Scores range from 0 (no response) to 4+ (very strong response). Predominant subclasses are bolded, co-dominant are underlined. For comparison purposes, western blot scores against PLA2R in HGE are included as well, as are ELISA titers (RU/mL) for anti-$PLA_2R$ total IgG. No $PLA_2R$ epitope data was collected from sample MN-12. Totals reported as *n* (%).

| | Subclass response per sample, western blot | | | | |
|---|---|---|---|---|---|
| | IgG1 | IgG2 | IgG3 | IgG4 | ELISA |
| MN-01, HGE | 2+ | 0 | 0 | **4+** | 188.5 |
| CysR | 4+ | 0 | 0 | 4+ | |
| CTLD1 | 0 | 0 | 0 | **3+** | |
| CTLD4-8 | 0 | 0 | 0 | **4+** | |
| MN-02, HGE | 2+ | 0 | **3+** | 1+ | 18.52 |
| CysR | 3+ | 0 | **4+** | 1+ | |
| CTLD1 | 0 | 0 | 0 | 0 | |
| CTLD4-8 | 0 | 0 | **1+** | 0 | |
| MN-03, HGE | 2+ | 2+ | 3+ | **4+** | 85.63 |
| CysR | 2+ | 0 | 0 | **4+** | |
| CTLD1 | 0 | 0 | 0 | **2+** | |
| CTLD4-8 | 0 | 0 | 0 | 0 | |
| MN-04, HGE | 2+ | 0 | 2+ | **3+** | 326.3 |
| CysR | 2+ | 0 | 2+ | **4+** | |
| CTLD1 | 0 | 0 | 0 | 0 | |
| CTLD4-8 | 0 | 0 | 0 | 0 | |
| MN-05, HGE | 3+ | 0 | 3+ | **4+** | 493.4 |
| CysR | 2+ | 0 | 4+ | 4+ | |
| CTLD1 | 0 | 0 | 0 | **1+** | |
| CTLD4-8 | 0 | 0 | 0 | **1+** | |
| MN-06, HGE | 1+ | 2+ | 1+ | **4+** | 148.4 |
| CysR | 0 | 0 | 0 | **2+** | |
| CTLD1 | 0 | 0 | 0 | **1+** | |
| CTLD4-8 | 0 | 0 | 1+ | 1+ | |
| MN-07, HGE | 1+ | 0 | 1+ | **2+** | 24.89 |
| CysR | 0 | 0 | 0 | 0 | |
| CTLD1 | 0 | 0 | 0 | 0 | |
| CTLD4-8 | 0 | 0 | 0 | 0 | |
| MN-08, HGE | 2+ | 1+ | 1+ | **4+** | 13.67 |
| CysR | 0 | 0 | 0 | **3+** | |
| CTLD1 | 0 | 0 | 0 | 0 | |
| CTLD4-8 | 0 | 0 | 0 | 0 | |
| MN-09, HGE | 1+ | 1+ | 1+ | **3+** | 146.2 |
| CysR | **3+** | 0 | 0 | 1+ | |
| CTLD1 | 0 | 0 | 0 | 0 | |
| CTLD4-8 | 0 | 0 | 0 | 0 | |

(Continued)

| Subclass response per sample, western blot (continued) | | | | | |
|---|---|---|---|---|---|
| | IgG1 | IgG2 | IgG3 | IgG4 | ELISA |
| MN-10, HGE | 3+ | 2+ | 4+ | 4+ | 355.4 |
| CysR | 3+ | 0 | 3+ | **4+** | |
| CTLD1 | 0 | 0 | 2+ | **4+** | |
| CTLD4-8 | 0 | 0 | 1+ | **4+** | |
| MN-11, HGE | 2+ | 1+ | 1+ | **3+** | 62.96 |
| CysR | **3+** | 0 | 0 | 2+ | |
| CTLD1 | 0 | 0 | 0 | 0 | |
| CTLD4-8 | 0 | 0 | 0 | 0 | |
| MN-13, HGE | 0 | 0 | 2+ | 2+ | 58.87 |
| CysR | n/a | n/a | 3+ | 3+ | |
| CTLD1 | n/a | n/a | 0 | 0 | |
| CTLD4-8 | n/a | n/a | **2+** | 1+ | |
| Total CysR | 8/11 (72.7) | 0/11 (0) | 5/12 (41.7) | 11/12 (91.7) | |
| Total CTLD1 | 0/11 (0) | 0/11 (0) | 1/12 (8.3) | 5/12 (41.7) | |
| Total CTLD4-8 | 0/11 (0) | 0/11 (0) | 4/12 (33.3) | 5/12 (41.7) | |

**Agreement between IgG against full-length $PLA_2R$ and epitopes**

We used western blot to assess the reactivity of IgG subclasses in serum against full-length $PLA_2R$ found in HGE, and compared it to reactivity against the three $PLA_2R$ epitopes (Table 14). We used Cohen's kappa to measure concordance between western blot scores in the same methods and scoring criteria as outlined previously (Viera & Garrett, 2005). Overall, the strongest agreement between autoantibodies directed against full-length $PLA_2R$ and individual epitopes was observed against CysR: IgG1 ($\kappa = 0.022$) exhibited slight agreement between the two targets, whereas IgG3 ($\kappa = 0.342$) and IgG4 ($\kappa = 0.231$) exhibited fair agreement. Autoantibodies against epitopes CTLD1 and CTLD4-8 exhibited slight agreement (from $\kappa = 0.057$ to 0.127) for reactivity with subclasses IgG3 and IgG4; IgG1 did not react with either of these epitopes. We found IgG2 to be completely unreactive with all $PLA_2R$ epitopes, and thus exhibited no concordance with reactivity to the full-length molecule.

We also compared the reactivity of circulating IgG subclasses against $PLA_2R$ epitopes with biopsy immunofluorescence data for corresponding IgG subclasses. Curiously, biopsy results exhibited considerably more agreement with western blot results for full-length $PLA_2R$, than with any of the epitopes. While several epitopes exhibited fair agreement with biopsy (CysR for IgG1; CysR, CTLD1 for IgG3; CTLD1, CTLD4-8 for IgG4), most notably anti-CysR IgG4 exhibited more disagreement with biopsy immunofluorescence for IgG4 than expected due to chance ($\kappa = -0.263$), especially surprising as anti-CysR IgG4 exhibited fair agreement with reactivity against full-length $PLA_2R$.

**Table 14, Concordance rating between $PLA_2R$, epitopes, and biopsy.** We determined the degree of concordance between IgG subclass reactivity to $PLA_2R$ epitopes, full-length $PLA_2R$ in HGE, and biopsy immunofluorescence. Results exhibiting fair agreement or more ($\kappa \geq 0.21$) are bolded. For statistical analysis we used scoring criteria as outlined in Table 6.

| Subclass | Full-length $PLA_2R$ | | Biopsy | |
|---|---|---|---|---|
| | Kappa | Std. error | Kappa | Std. error |
| IgG1 | | | | |
| CysR | 0.022 | 0.156 | 0.172 | 0.191 |
| CTLD1 | 0.000 | 0.000 | 0.000 | 0.000 |
| CTLD4-8 | 0.000 | 0.000 | 0.000 | 0.000 |
| IgG2 | | | | |
| CysR | 0.000 | 0.000 | 0.000 | 0.000 |
| CTLD1 | 0.000 | 0.000 | 0.000 | 0.000 |
| CTLD4-8 | 0.000 | 0.000 | 0.000 | 0.000 |
| IgG3 | | | | |
| CysR | **0.342** | 0.151 | 0.122 | 0.186 |
| CTLD1 | 0.127 | 0.113 | 0.020 | 0.024 |
| CTLD4-8 | 0.057 | 0.059 | -0.047 | 0.094 |
| IgG4 | | | | |
| CysR | **0.231** | 0.214 | -0.263 | 0.157 |
| CTLD1 | 0.067 | 0.053 | 0.043 | 0.039 |
| CTLD4-8 | 0.067 | 0.055 | 0.043 | 0.040 |

## Correlation between anti-CysR IgG and clinical characteristics

We analyzed correlation between IgG subclass responses against CysR and patient clinical characteristics, particularly because response against CysR exhibited the greatest concordance with IgG subclass responses as a whole. Using Kendall rank correlation coefficient, we found significant correlation between anti-CysR IgG subclasses and the following (Table 15): for IgG1 with age ($\tau = 0.52$, $P = 0.03$); for IgG3 negatively with serum creatinine ($\tau = -0.43$, $P = 0.04$) and negatively with C3 ($\tau = -0.41$, $P = 0.05$); for IgG4 with serum anti-$PLA_2R$ ($\tau = 0.52$, $P = 0.03$). Correlation between IgG3 with C3 staining, and IgG4 with levels of serum anti-$PLA_2R$ IgG were also observed.

**Table 15, Correlation between clinical data and CysR reactivity.** We analyzed significant correlation between anti-CysR IgG subclass responses and patient clinical characteristics. We were unable to calculate a correlation coefficient for IgG2 as it exhibited no reactivity with CysR. For analysis, Caucasian ethnicity = 0, and Asian = 1; for sex, male = 0 and female = 1. Bolded values indicates significance at $P \leq 0.05$.

| | **Kendall rank correlation coefficient (τ)** | | | | | |
|---|---|---|---|---|---|---|
| | IgG1 | | IgG2 | IgG3 | | IgG4 | |
| Age | **0.52** | $P = 0.03$ | n/a | 0.40 | | 0.34 | |
| Sex[1] | -0.22 | | n/a | 0.00 | | 0.05 | |
| Ethnicity[2] | 0.23 | | n/a | -0.26 | | -0.34 | |
| Clinical information | | | | | | | |
| Serum creatinine | 0.31 | | n/a | **-0.43** | $P = 0.04$ | -0.19 | |
| Serum albumin | -0.18 | | n/a | 0.32 | | -0.08 | |
| UPCR | 0.27 | | n/a | -0.17 | | -0.29 | |
| Anti-$PLA_2R$ | 0.19 | | n/a | 0.24 | | **0.49** | $P = 0.03$ |
| Immunohistological staining | | | | | | | |
| C3 | 0.05 | | n/a | **-0.41** | $P = 0.05$ | -0.19 | |
| C1q | -0.24 | | n/a | -0.13 | | -0.40 | |
| Total IgG | -0.06 | | n/a | 0.00 | | -0.07 | |
| Months after Bx | -0.08 | | n/a | -0.15 | | -0.03 | |

## DISCUSSION

Use of autoantibodies to $PLA_2R$ as a diagnostic and prognostic biomarker has increased considerably since its discovery in 2009. Research groups have shown IgG4 predominance in circulation (Hofstra, et al., 2012) and in glomerular deposits (Doi, Mayumi, Kanatsu, Suehiro, & Hamashima, 1984), though so far studies have lacked a comprehensive comparison of all IgG subclasses in both locations. In the present we have reinforced that knowledge by specifically showing concordance between levels of anti-$PLA_2R$ IgG subclasses in serum and IgG subclass levels within glomerular deposits as indicated by renal biopsy. We have also demonstrated concordance between levels of particular circulating IgG subclasses against full-length $PLA_2R$ as against the CysR, CTLD1, and CTLD4-8 domains, each of which harbors a unique epitope.

A potential concern about our results is that immunofluorescence for IgG subclasses, as indicated by the routine biopsy reports, might represent multiple IgG specificities, some of which may be directed against antigens other than $PLA_2R$. While this may appear to limit our conclusions comparing circulating and deposited IgG, there is in fact evidence that IgG in glomerular deposits is predominantly and specifically anti-$PLA_2R$. Acid elution from biopsy tissue has demonstrated that anti-$PLA_2R$ IgG4 can be specifically eluted from biopsies of primary MN patients, but not from those of secondary MN patients or other disease controls (Beck, et al., 2009). Several studies have shown precise co-localization of the $PLA_2R$ antigen and IgG4. However, neither of these observations preclude the existence of antibodies directed against other glomerular

antigens. A rare case involving a monoclonal IgG3κ anti-$PLA_2R$ associated with recurrent MN is instructive, in that subepithelial deposits in both the kidney allograft and the native kidney contained only IgG3κ (Debiec, et al., 2012). Had there been immunoglobulins directed against antigens other than $PLA_2R$, it is extremely unlikely that they would have also been restricted to this subclass and particular light chain. Using conclusions drawn from these two studies, we consider the biopsy staining results for IgG subclasses to be a good approximation of IgG specific against $PLA_2R$, and thus in our study we have assessed biopsy results under this assumption.

**Anti-$PLA_2R$ directed against HGE**

As measured by western blot, anti-$PLA_2R$ levels within the patient serum samples conformed to subclass distributions typically exhibited in serum of primary MN patients. Our observation of circulating IgG4 predominance, and a weaker presence of circulating IgG1 and IgG3, is also consistent with previous conclusions that glomerular deposits in primary MN contain chiefly IgG4, potentially with weaker IgG1 and IgG3 staining depending on the stage of disease (Huang, et al., 2013). Generally, IgG subclass distribution measured by western blot, excluding IgG2, was concordant with subclass staining in biopsy reports. IgG2 presented as the weakest subclass when measured by both, but was more strongly detected in glomerular deposits: there was a 46.2% positive detection rate in western blot and 76.9% positive detection rate in biopsy (Tables 6 & 8), which represented the lowest rate for each group. Biopsy immunofluorescence usually scored higher for all samples compared to western blot (Figures 10 & 11) This

discrepancy is not surprising since podocyte $PLA_2R$ "can be detected in antibody-negative patients," owing to the higher sensitivity for detecting $PLA_2R$ antigen compared to its corresponding autoantibody (Ronco & Debiec, 2014), in addition to detecting deposited IgG in patients who have entered immunological remission (Beck, et al., 2009). Because the biopsy reflects IgG deposits accumulated over a period of several months to years, it is expected to score higher than a serum sample which only represents a single—and thus immunologically weaker—time point, especially when a patient goes into remission. We alleviated this inherent disagreement by grouping western blot and biopsy scores into strength categories (Table 5) and then performed concordance analysis. Based on these results, we conclude that for most (but not all) patients with primary MN, circulating IgG were fairly reflective of IgG deposited in glomeruli over the entire time course of a patient's disease.

Notably, there were three cases measured by western blot in which circulating IgG4 was not the predominant subclass: IgG3 was dominant in one case (MN-02), and co-dominant with IgG4 in two cases (MN-10 and MN-13). The predominance of circulating IgG3 was unexpected, as its corresponding biopsy exhibited IgG4 predominance. There is significant positive correlation between circulating IgG4 and anti-$PLA_2R$ total IgG, and MN-02 was also reported to have the lowest anti-$PLA_2R$ titer among all patient samples, offering a serological explanation for the weak reactivity of IgG4 by western blot. Additionally, the western blot is performed using a serum sample representing a single time point during the patient's immunological course of disease, whereas biopsy immunofluorescence is the result of accumulated immune complex

deposition over a much longer period of time. Another hypothetical explanation for the lack of IgG4 predominance may be due to an unrecognized secondary cause of MN or coincidental condition, one which may not have been clearly indicated in the patient's biopsy report. In such cases, inflammatory diseases or malignancies produce an immune response which may divert antibody production away from the IgG4 subclass. Cell-mediated immunity conferred by activation of Type 1 T-helper cell ($T_h1$) populations is directed against bacterial and viral infections (Kuby, 1997), and also against tumor cells (Hung, et al., 1998). In contrast, humoral responses driven by predominately by the $T_h2$ population leads to increased immunoglobulin production, especially of the IgG4 subclass, and is more associated with primary MN (Hirayama, et al., 2002). Activation of one T-helper cell subset can inhibit the other (Kuby, 1997), which provides a possible explanation for circulating IgG3 predominance and not IgG4, though the evidence is mostly circumstantial for this particular case. Supporting these ideas, we also performed western blot against HGE using four patient serum samples featuring malignancy-induced secondary MN. In three samples we detected prominent circulating IgG1, IgG3, in addition to strong IgG4, and in the fourth sample, predominantly IgG4 and a lesser amount of IgG3 (data not shown). More data should be obtained from MN patients with malignancy-associated cases to derive more insight regarding the distribution of circulating IgG subclasses.

So far, our findings provide more insight regarding IgG subclass distribution, though conclusive data for their pathological roles in primary MN is still lacking. Previous studies have shown that IgG1 and IgG3 are able to activate the classical

complement cascade, and positively correlate with C1q staining (whereas IgG4 correlates negatively with classical complement component C1q) which implies that these subclasses may cause damage via complement in earlier stages of primary MN progression (Huang, et al., 2013). This idea is further grounded in fetomaternal alloimmune MN, wherein cases exhibit both IgG1 and IgG4 anti-NEP predominance, and complement-fixing IgG are additionally required to cause proteinuria (Vivarelli, et al., 2015). We found statistically significant negative correlation between circulating IgG4 and glomerular C1q staining (Table 10), a relationship that Huang et al. also established with glomerular IgG4. As IgG4 is unable to activate complement by the classical pathway, there may be a physiological foundation for these results. In our study, however, we were unable to establish significant correlation between either IgG1 or IgG3 and C1q. Therefore, it is difficult to make further assertions concerning the role of complement activation in primary MN based solely on our results.

**Anti-$PLA_2R$ directed against epitopes**

A majority of autoantibodies were reactive against CysR (91.7% total samples were positive for anti-CysR IgG of any subclass), and the rest exhibited minor reactivity against CTLD1 and CTLD4-8 (Table 13). This is consistent with previous findings in which the immunodominant epitope was contained within the CysR region (Fresquet, et al., 2015). However, in a different study reactivity with anti-$PLA_2R$ was only established with an epitope construct encompassing CysR-FNII-CTLD1; reactivity was completely abolished when CTLD1 was removed from the construct, possibly due to the latter

epitope influencing conformation via disulfide bonding (Kao, Lam, Waldman, Glassock, & Zhu, 2015). We were able to detect reactivity to the isolated CysR epitope, and this may be due to the fact that in our experiments serum was typically diluted between 1:25 and 1:100, compared to the 1:1000 dilution used for most experiments in Kao et al. (1:100 was our default dilution for all experiments, decreasing down to 1:25 in some cases that were undetectable at the former dilution). While some of our serum samples were also reactive with $PLA_2R$ epitopes at higher dilutions, most required at least 1:100 dilution or lower for detection by western blot (data not shown).

In most cases, we have also shown circulating autoantibodies reactive with individual epitopes to be IgG4 predominant and most widespread: IgG4 reactive against CysR was found in almost all serum samples, and reactive against CTLD1 and CTLD4-8 in approximately half of all samples (Table 13). Additionally, serum anti-CysR IgG3 and IgG4 (the two most reactive subclasses overall against $PLA_2R$ epitopes) exhibited fair agreement with levels of IgG3 and IgG4 reactive against full-length $PLA_2R$, indicating that for these two subclasses, the immunogenicity of CysR as well as anti-CysR subclass distribution mirrors that of the full-length molecule (Table 14). In contrast, reactivity against CTLD1 and CTLD4-8 for both IgG3 and IgG4 exhibited only slight agreement with reactivity against full-length $PLA_2R$; these epitopes may not be as effective an indicator of primary MN if they were used in isolation. Nevertheless, with the exception of a single case (MN-06) autoantibodies reactive against CTLD1 and CTLD4-8 were never detected unless anti-CysR IgG was also present, and even in the single anomalous case anti-CTLD4-8 IgG was only weakly positive. Interestingly, anti-CysR IgG4 was not

the predominant subclass in four cases: IgG4 exhibited co-dominance with anti-CysR IgG1 in one case (MN-01), was subdominant to IgG1 in two more cases (MN-09 and MN-11), and subdominant to IgG3 in another (MN-02). IgG1 predominance in MN-09 and MN-11 was unexpected, as this was not reflected at all in western blot for full-length $PLA_2R$—in fact, in these experiments IgG1 reactivity was rated "very weak" and "moderate," respectively. At present, we cannot offer a satisfactory explanation for this discrepancy. On the other hand, the unusual IgG3 predominance in MN-02 was more expected as this was also observed in the same sample against full-length $PLA_2R$, reinforcing the idea of CysR primacy.

To conclude, predominance of IgG4 reactivity towards $PLA_2R$ domains containing epitopes CysR, CTLD1, and CTLD4-8 carries over from the full-length molecule, and overall CysR epitope garners the strongest immune responses. We observe both IgG3 and IgG4 to exhibit concordance when reactive against CysR and the full-length $PLA_2R$, and we also observe circulating autoantibody reactivity against CTLD1 and CTLD4-8 to usually occur when there is also stronger anti-CysR reactivity. CysR has potential as an adequate marker primary MN, especially when used to observe IgG4 predominance. Based on the lack of universal reactivity to CysR demonstrated by our data, we still urge caution against using anti-CysR IgG4 exclusively to monitor immunologic disease activity of primary MN in general. Applied use of anti-CysR as a biomarker to monitor treatment of primary MN should complement, and not replace, existing standards.

**Limitations, future directions, and final remarks**

As with most statistical analyses, we need to address the possibility of type I and type II errors in our correlations between IgG subclasses measured in western blot and clinical characteristics. With some clinical features, we occasionally detected significant correlations (based on $P \leq 0.05$ for significance) that were either unexpected, unconvincing, or with poor theoretical plausibility. IgG1 and IgG3 were correlated with Caucasian ethnicity, and IgG3 negatively with C3 staining (Table 10). For ethnicity, though MN tends to affect the Caucasian population more strongly (Salant & Cattran, 2010), 10/13 of our patient samples were also Caucasian, the limited number of subjects from other ethnic groups weakens our result. The negative correlation between circulating IgG3 and C3 staining was another curious case, as one would expect high levels of complement-activating IgG3 to also be associated with high levels of complement components, as has been established in previous studies (Huang, et al., 2013). We also explored options to reduce possible false positive errors using Bonferroni correction, which rendered all of our results non-significant due to increased stringency for the *P*-value requirement. This method also increases false negative rates, and its usage is cautioned unless a large number of multiple comparisons testing a single universal null hypothesis are involved (Armstrong, 2014). Instead, a more effective and necessary method may be to simply use larger sample groups, which could also improve the quality of all statistical findings. It is worth mentioning, however, that larger sample groups may exacerbate the problem of biopsy scores being obtained from different pathologists. This would produce an additional source of heterogeneity which may have affected our results

in this study. A final concern regards our use of semi-quantitative scoring on a scale of 0 through 4+ for western blot, which may limit our choices in statistical analysis and their robustness. Ideally, more quantitative results for western blot can be obtained by performing densitometry analysis to produce continuous values. However, this technique is more sensitive to variation—our western blots were performed over a period of time without a reliable internal control, and as such we are not able to use this technique for a post-hoc analysis.

Evaluating immunogenicity of specific $PLA_2R$ epitopes involves some additional challenges. In our experiment, we did not test for reactivity against CTLD2 or CTLD3 due to limited reactivity observed in previous experiments (data not shown), nor against any individual epitopes within CTLD4-8 due to technical issues expressing these domains in isolation. As conformational epitopes of $PLA_2R$ are known to exist, one can speculate that testing IgG subclass reactive against different sequential combinations may reveal epitopes previously hidden, though this may also introduce new and non-physiological epitopes that would have been undetected natively. Related to this issue, we had attempted blotting HGE directly onto nitrocellulose membrane, without intervention from the denaturing step (heating in an excess of SDS) associated with SDS-PAGE. We had hoped to find whether anti-$PLA_2R$ IgG directed against HGE would differ depending on presence of denaturing conditions, but were unable to observe any differences in preliminary experiments (data not shown).

Detecting serum anti-$PLA_2R$ autoantibody levels has proven to be a valuable tool in evaluating disease and guiding patient treatment. With increased prominence and

availability of commercial immunofluorescence kits and ELISA assays, we are now able to quickly and accurately measure levels of circulating anti-$PLA_2R$, and possibly reducing the need for intrusive biopsies. Regardless, when testing patients for circulating anti-$PLA_2R$, we are still unable to detect autoantibodies in approximately 20% to 30% of cases. This may be due to differences between assays, patients achieving immunological remission, or primary MN caused by a different antigen entirely. Partly due to these reasons, some researchers urge caution before elevating anti-$PLA_2R$ titers to the status of a diagnostic end-all (Hofstra & Wetzels, 2012). In particular, the cohort of primary MN patients with missing reactivity to $PLA_2R$ have prompted researchers to speculate about as-of-yet unknown glomerular antigens and their involvement in primary MN. Very recently thrombospondin type-1 domain-containing 7A (THSD7A), a transmembrane podocyte protein similar to $PLA_2R$, was identified by our laboratory, in collaboration with two European groups, to be another antigenic target that can cause immune complex deposition and primary MN (Tomas, et al., 2014). In this study, less than 10% of patients with primary MN but without reactivity to $PLA_2R$ contained anti-THSD7A autoantibodies, leaving the possibility of yet more undiscovered antigenic targets in primary MN.

The time delay experienced between immunological and clinical markers of the disease posed a challenge to the analysis and interpretation of our data. Since our samples were collected as individual time-points in relation to their biopsies, we did not have thorough descriptions of the patients' disease progression—the overall lack of correlation between patient clinical characteristics and serum anti-$PLA_2R$ IgG subclass levels may be

attributed to this fact, as may the occasional discrepancy between reactivity against full-length $PLA_2R$ and its epitopes. Whether such disagreement between clinical conditions and anti-$PLA_2R$ levels were truly due to lack of correlation, or due to mismatched phases of immunological or clinical disease is difficult to conclusively determine without additional data and more thorough clinical history. The high affinity of anti-$PLA_2R$ autoantibodies for podocyte $PLA_2R$ can explain some of these effects for patients with early stages of primary MN. The absorption of circulating IgG from plasma at a faster rate than that by which it can be produced may lead to a circumstance in which there is no detectable circulating anti-$PLA_2R$ while IgG- and antigen-containing deposits already exist within the biopsy. Once the immunological disease and antibody production increases, anti-$PLA_2R$ may then be more easily detected in circulation:

> "This could explain the clinical paradox of $PLA_2R$-positive biopsy specimens associated with absence of anti-$PLA_2R$ antibodies in the serum…These biopsy-positive, seronegative cases may, therefore, represent an early phase of…primary disease and in recurrent disease" (Fresquet, et al., 2015).

Alternatively, patients who have resolving immunologic disease but have not undergone clinical remission also exhibit proteinuria in absence of circulating anti-$PLA_2R$, and lead to discrepancies between autoantibody titers and clinical characteristics. This explanation had been previously evidenced in cases of primary MN patients with undetectable serum titers of anti-$PLA_2R$ but positive glomerular staining for the same, suggesting that symptoms may persist after autoantibody clearance from circulation due to long-term ultrastructural damage which had yet to be repaired (Debiec & Ronco, 2011). In light of this information, some of the discrepancies observed between IgG subclasses directed

against full-length $PLA_2R$, its epitopes, and immunofluorescence may be attributed to the progressing stages of filtration barrier damage and repair, as well as to rapid clearance and deposition of circulating autoantibodies.

Despite these challenges, anti-$PLA_2R$ has so far proven useful as a clinical guide for diagnosis and treatment of primary MN. We have now added to this body of knowledge by demonstrating an overall consistency of distribution for all subclasses between serum and tissue. We have also shown IgG4 predominance in reactivity to individual $PLA_2R$ epitopes, and despite previously described limitations these observations can serve to enhance the understanding of primary MN. By undertaking additional studies in the future incorporating more detailed patient history, clinical characteristics, as well as a larger cohort size, researchers and physicians in this field may hopefully codify more concise relationships between these biomarkers and apply this knowledge towards better treatment and care of patients with primary MN.

## APPENDIX

Results for western blot of all patient samples.

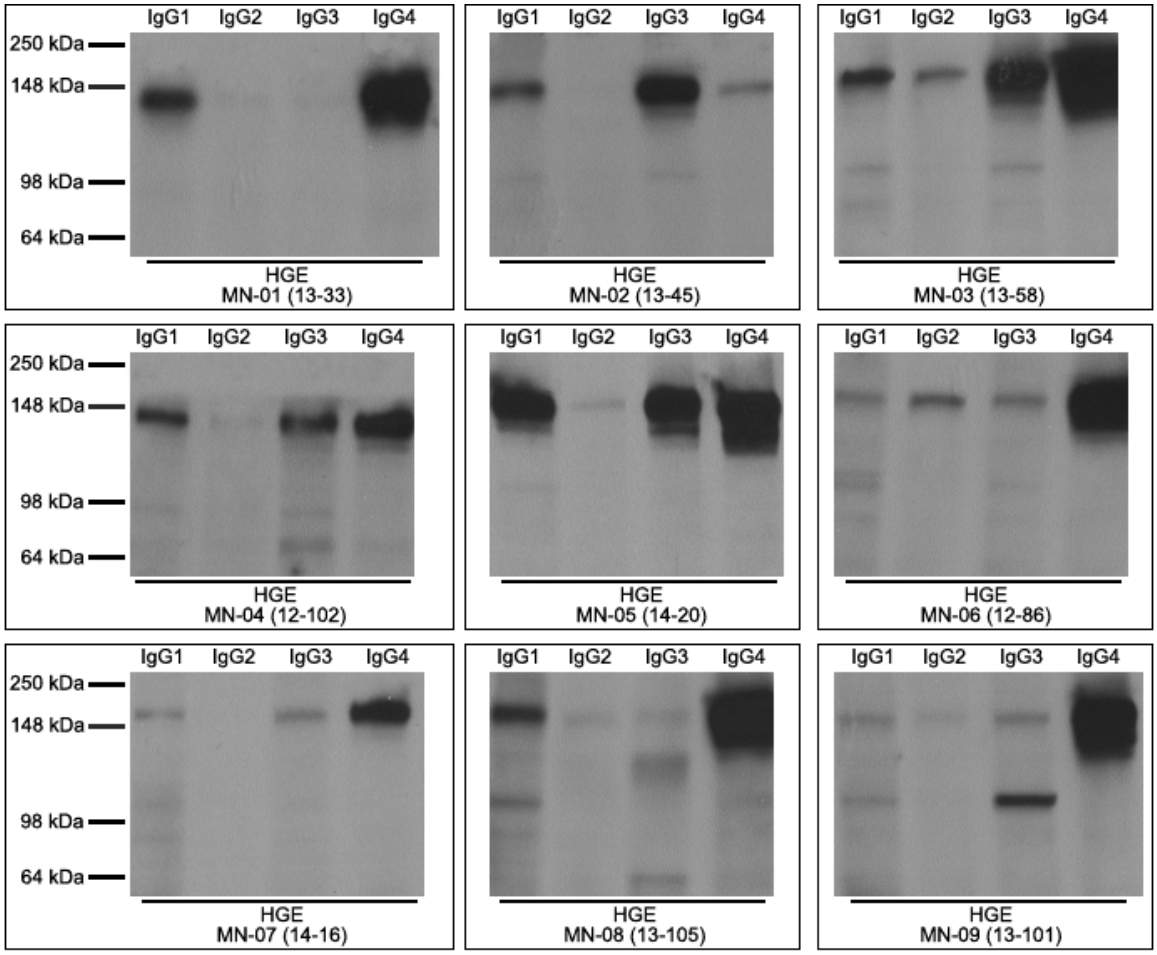
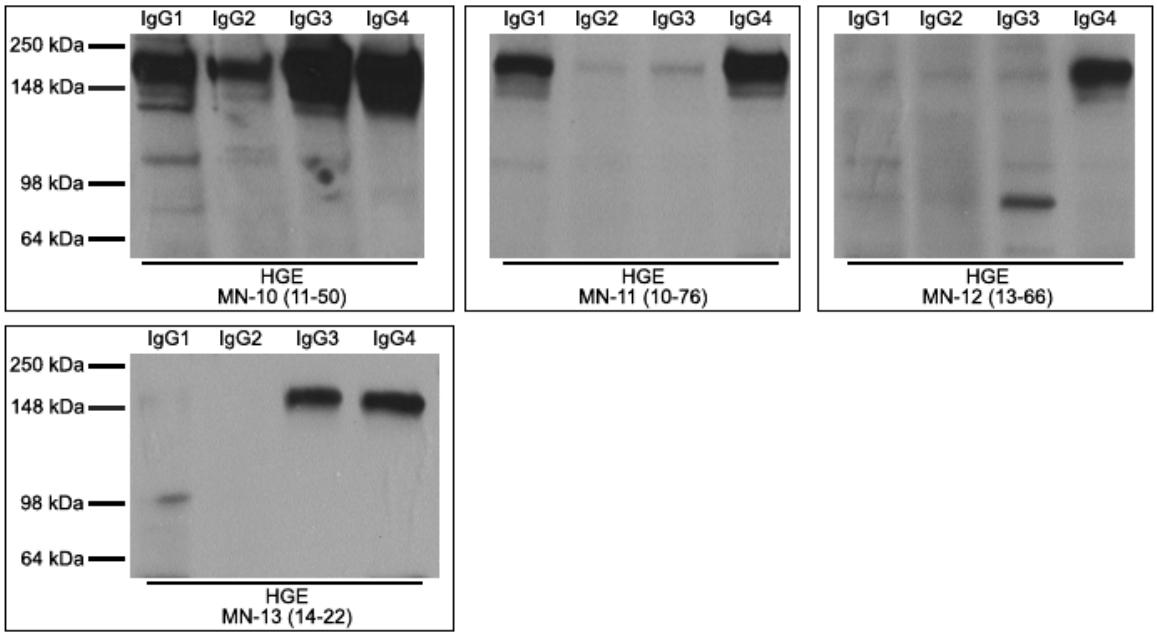



**Figure 13, All IgG subclass responses against HGE.** Western blot exposures of patient serum samples (indicated by sample number and corresponding biopsy report identifiers). HGE was probed with MN-positive serum (1:100 dilution), and recognized bands approximately corresponding to the size of $PLA_2R$ (~185 kDa). The blots pictured here were exposed for 30s each.

(Continued from previous section.)



Results for western blot of all samples, grouped by $PLA_2R$ epitope.

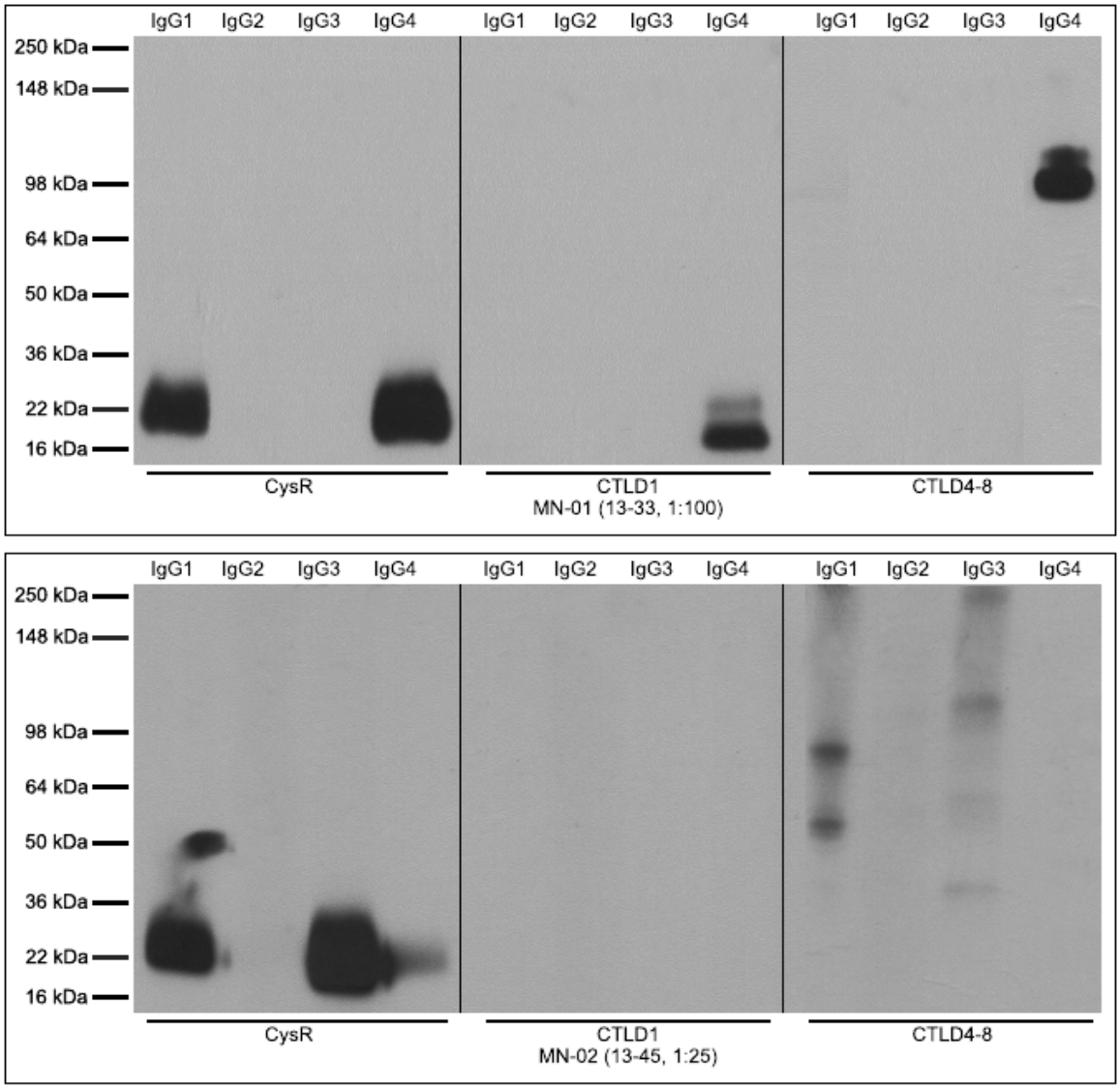
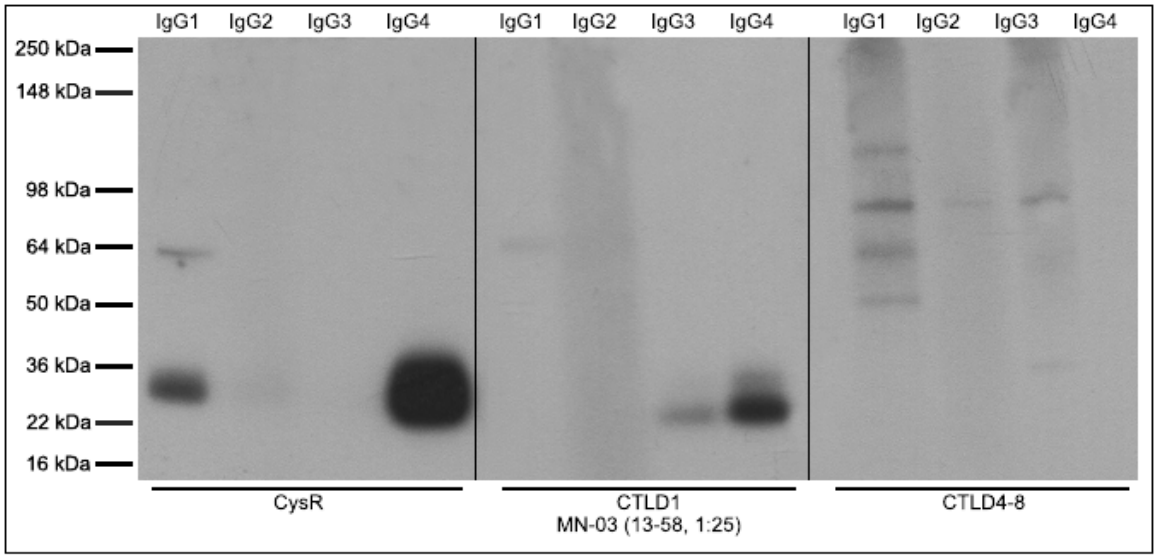
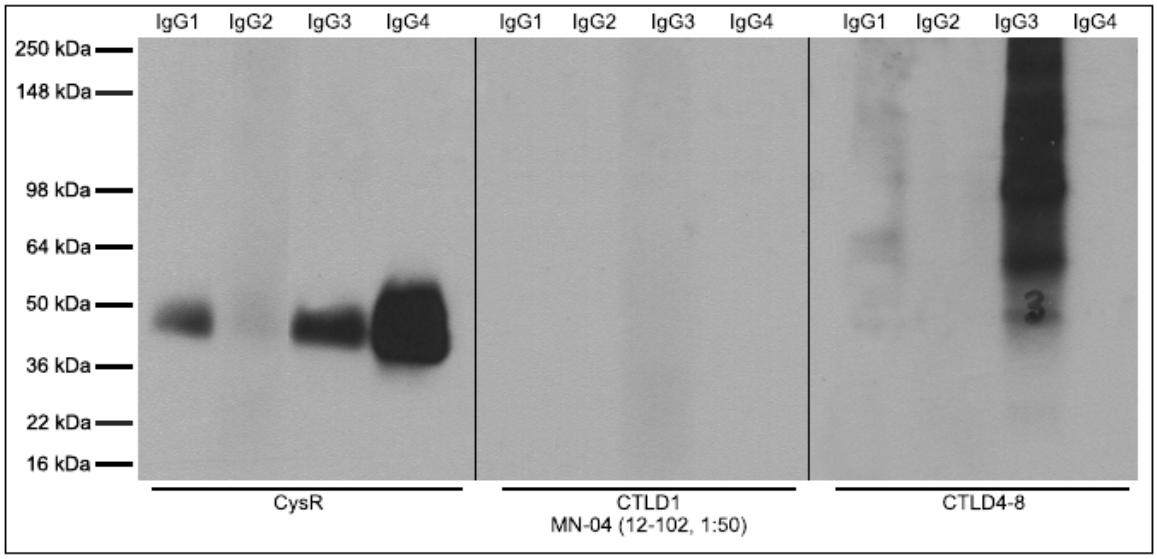



**Figure 14, All IgG subclass responses against $PLA_2R$ epitopes.** Western blot exposures of patient serum samples (indicated by sample number and corresponding biopsy report identifiers). Domains of PLA2R containing three epitopes constructs were probed with MN-positive serum (dilutions ranging from 1:25 to 1:100), and recognized bands approximately corresponding to the sizes of CysR (~20 kDa), CTLD1 (~20 kDa), and CTLD4-8 (~90 kDa). The blots pictured here were exposed for 30s each.

(Continued from previous section.)



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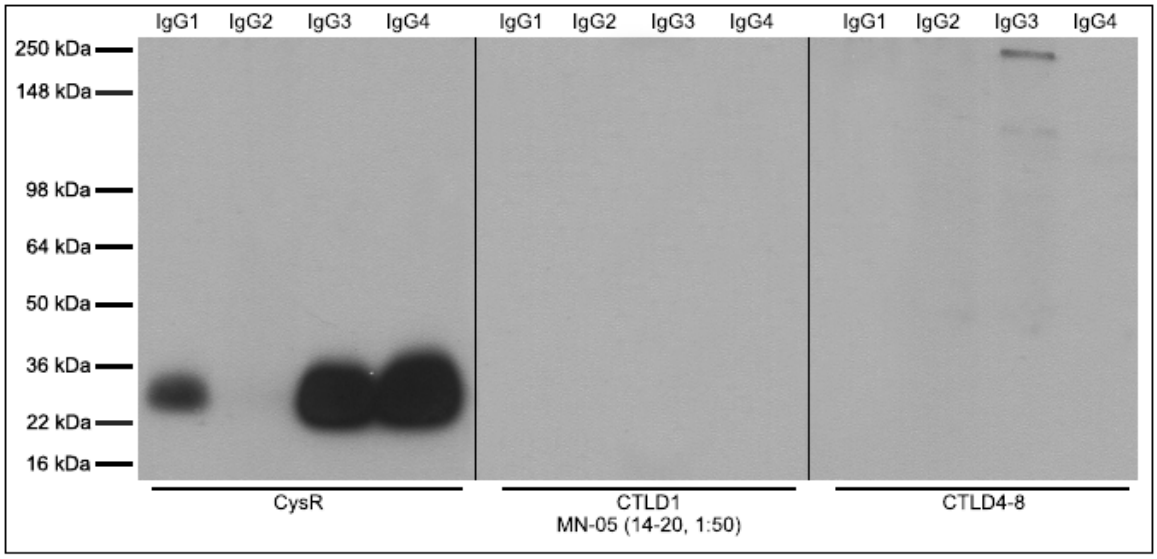


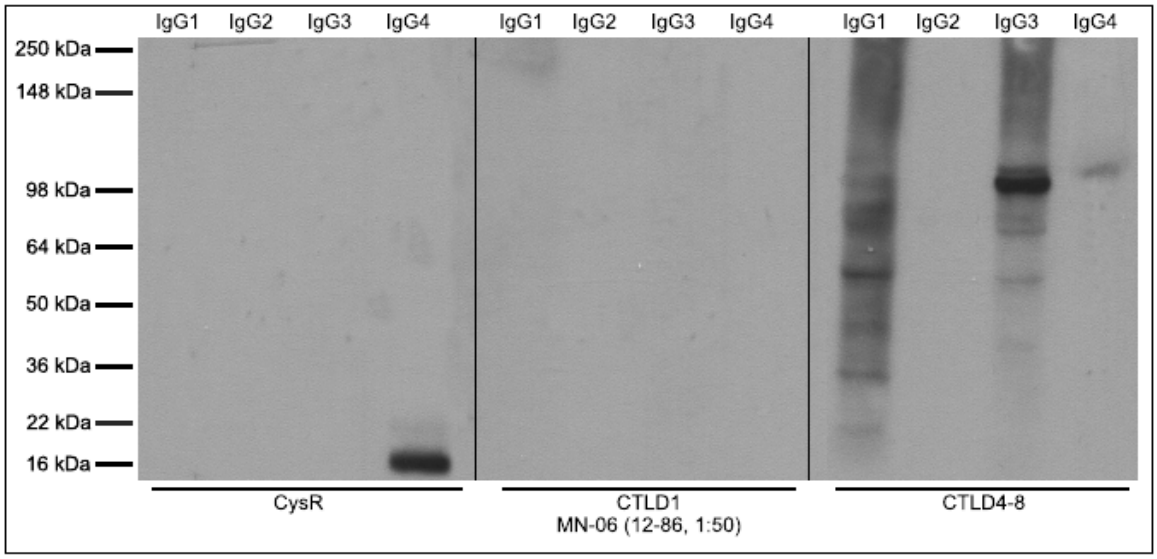

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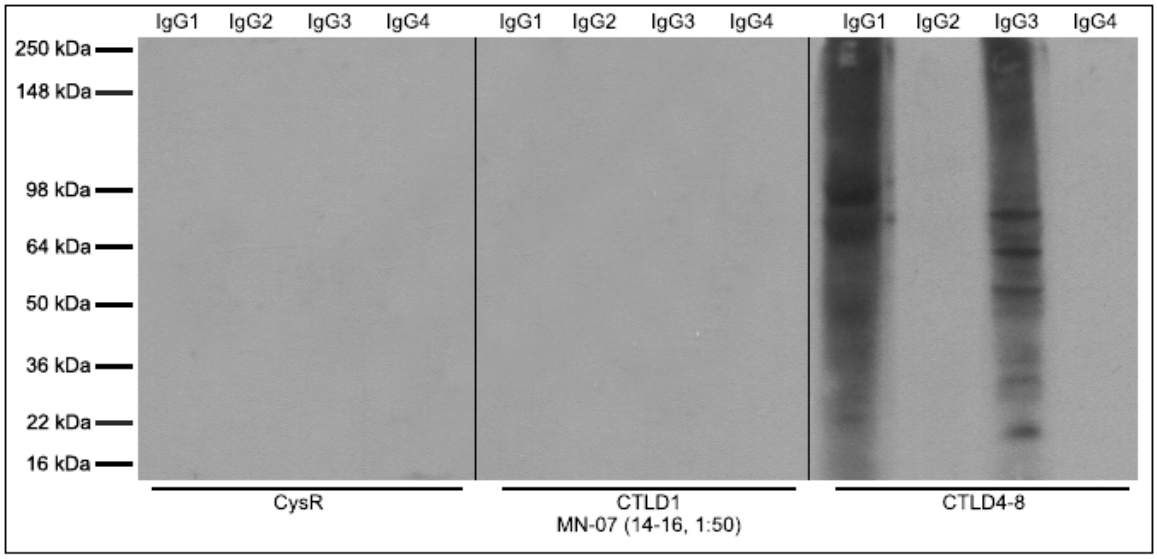


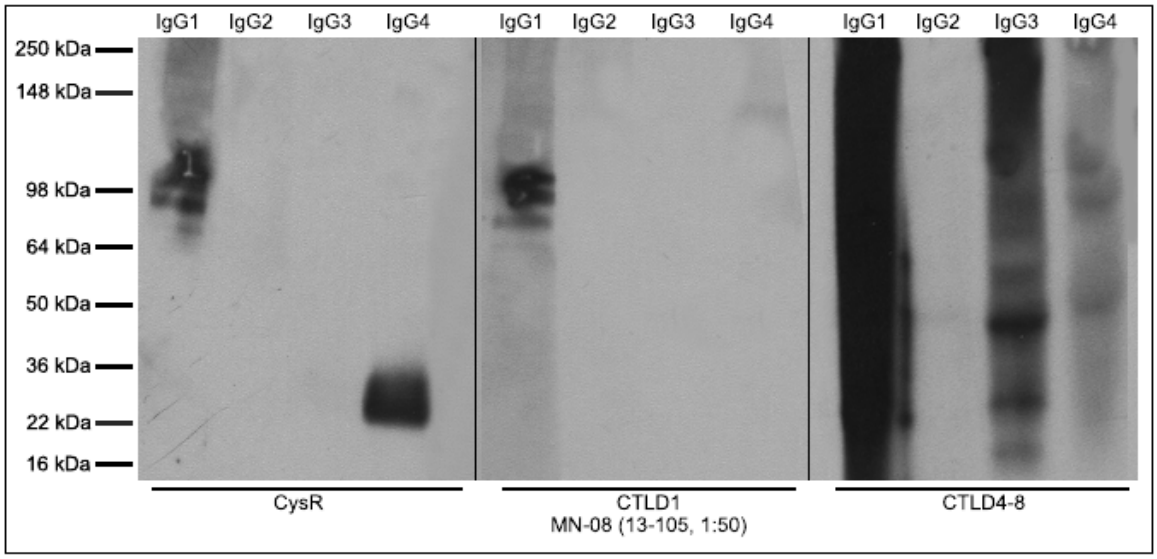

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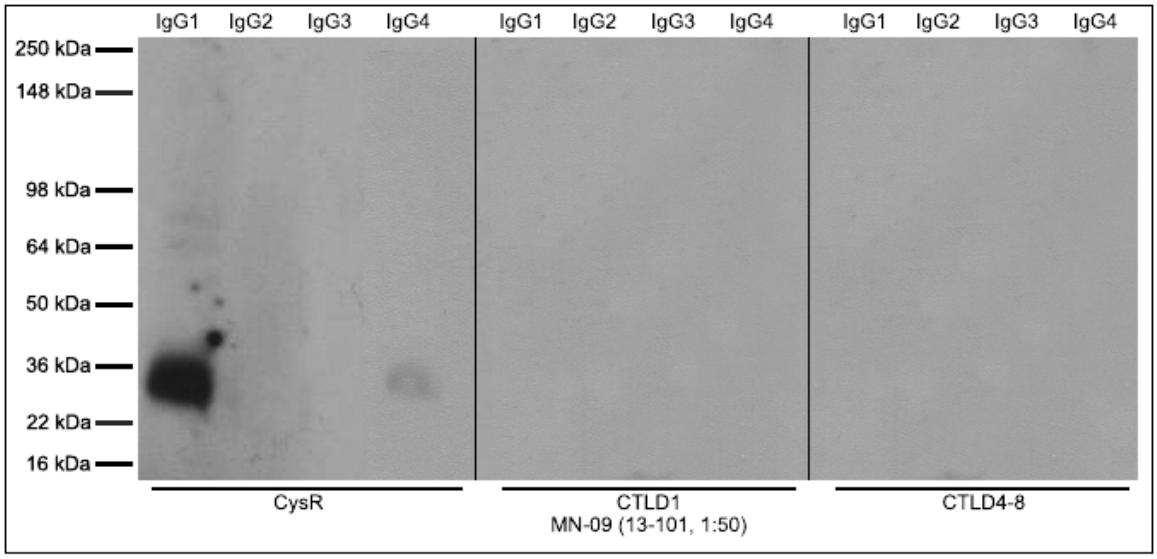


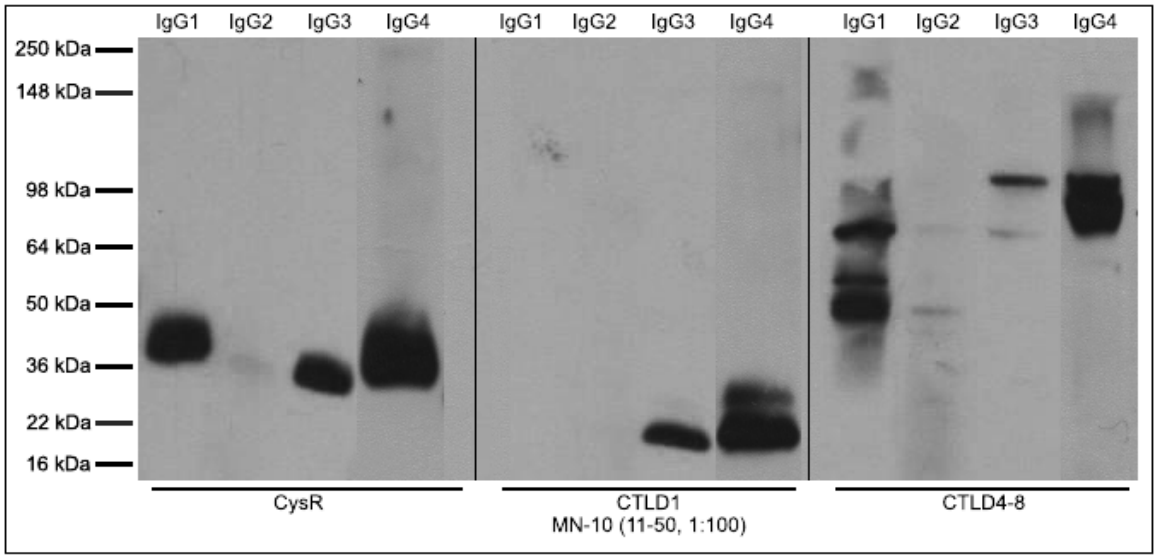

(Continued from previous section. Western blot was not performed with IgG1 and IgG2 for sample MN-13)

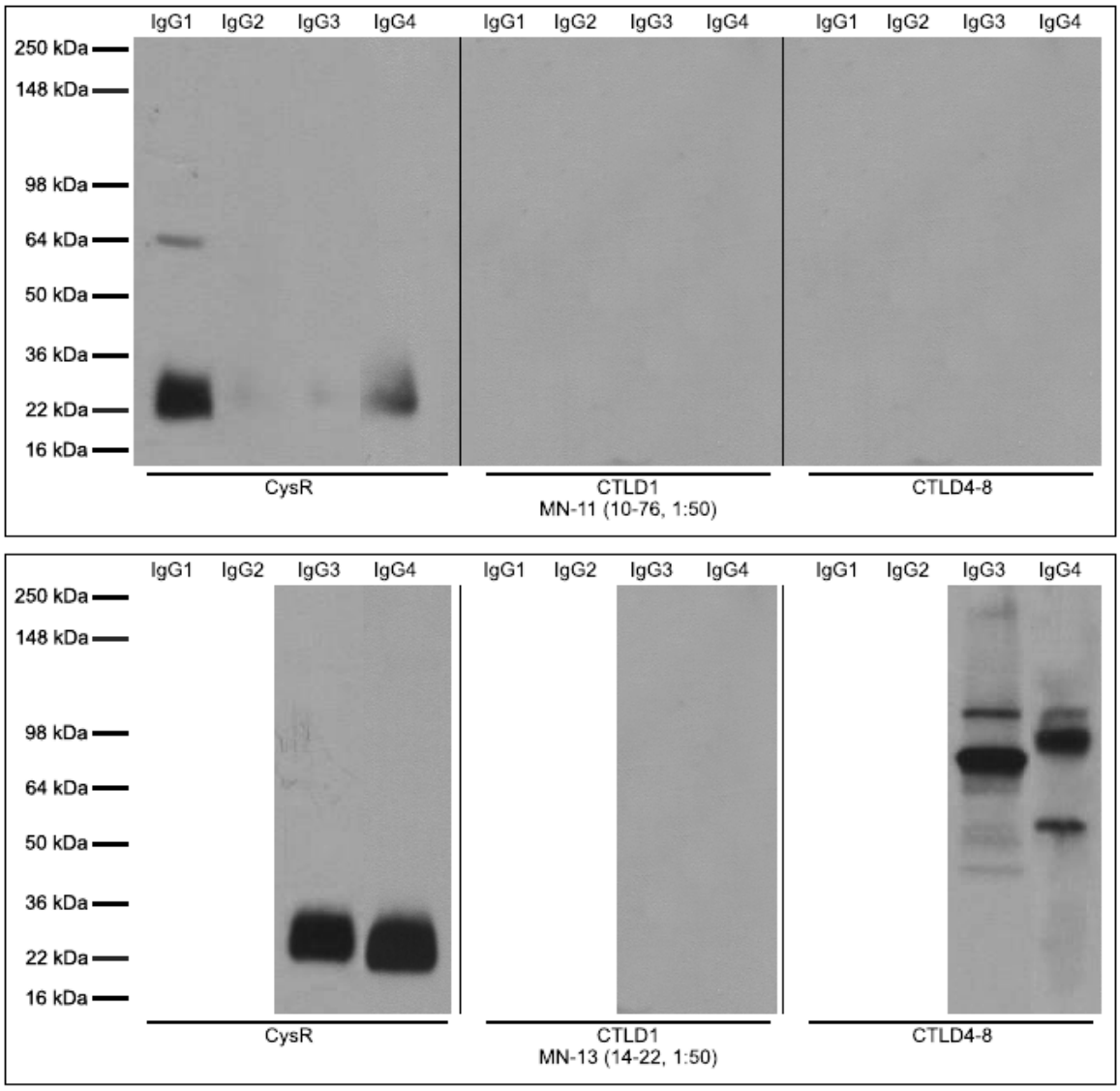

**Table 16, Clinical characteristics of patient samples.** Clinical information for each patient as indicated in the biopsy reports. [1]Serum creatinine (mg/dL). [2]Serum albumin (g/dL). [3]Serum anti-$PLA_2R$ as measured by ELISA (RU/mL). [4]Months elapsed after biopsy date that serum sample was taken (serum for MN-13 was taken before the corresponding biopsy).

| Sample | Age | Sex | Ethnicity | S. cr.[1] | S. albu.[2] | UPCR | Anti-$PLA_2R$[3] | C3 | C1q | Total IgG | Mos.[4] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **MN-01** | 70 | M | Caucasian | 1.95 | 1.7 | 8 | 188.5 | 3+ | 0+ | 4+ | 5.6 |
| **MN-02** | 71 | M | Caucasian | 1.25 | 4 | 9.5 | 18.52 | 2+ | 1+ | 4+ | 2.1 |
| **MN-03** | 63 | M | Caucasian | 1.7 | 3.2 | 11.1 | 85.63 | 2+ | 1+ | 4+ | 0.7 |
| **MN-04** | 79 | F | Caucasian | 1.4 | n/a | 3.13 | 326.3 | 2+ | 0.5+ | 4+ | 1 |
| **MN-05** | 57 | M | Caucasian | 1.02 | 2.2 | 8.2 | 493.4 | 2+ | 0+ | 4+ | 3 |
| **MN-06** | 34 | F | Caucasian | 0.75 | 2.2 | 8.4 | 148.4 | 3+ | 0+ | 4+ | 13 |
| **MN-07** | 34 | M | Caucasian | 3.02 | 2.4 | 9.87 | 24.89 | 3+ | 2+ | 4+ | 11.1 |
| **MN-08** | 53 | F | n/a | 0.7 | 3.7 | 5.3 | 13.67 | 0.5+ | n/a | 3+ | 4.7 |
| **MN-09** | 59 | F | Asian | 2.02 | 2.3 | 19.7 | 146.2 | 2+ | 0+ | 4+ | 1.5 |
| **MN-10** | 64 | F | Caucasian | 1.3 | 3.5 | 19 | 355.4 | 1+ | 0+ | 3+ | 19 |
| **MN-11** | 54 | M | Caucasian | 2.7 | 1.7 | 16 | 62.96 | 3+ | 1+ | 3+ | 3 |
| **MN-12** | 77 | M | Asian | 2.3 | n/a | 5.2 | 33.12 | 2+ | 0+ | 3.5+ | 4 |
| **MN-13** | 69 | F | Caucasian | 0.66 | 2.8 | 3.74 | 58.87 | 0+ | n/a | 3.5+ | -0.6 |

## LIST OF JOURNAL ABBREVIATIONS

Am J Pathol ..... American Journal of Pathology

Ann NY Acad Sci .....Annals of the New York Academy of Sciences

Clin Exp Immunol.....Clinical and Experimental Immunology

Clin Exp Nephrol ..... Clinical and Experimental Nephrology

Fam Med .....Family Medicine

Int J Epidemiol..... International Journal of Epidemiology

Intern Med.....Internal Medicine

J Am Soc Nephrol..... Journal of the American Society of Nephrology

J Clin Invest .....Journal of Clinical Investigation

J Clin Pathol.....Journal of Clinical Pathology

J Exp Med .....Journal of Experimental Medicine

Kidney Int ..... Kidney International

Modern Pathol.....Modern Pathology

Nephrol Dial Transpl ..... Nephrology Dialysis Transplantation

Nephron Clin Pract .....Nephron Clinical Practice

Neth J Med.....Netherlands Journal of Medicine

New Engl J Med..... New England Journal of Medicine

Ophthal Physl Opt.....Ophthalmic and Physiological Optics

Pathol Annu .....Pathology Annual

Pediatr Nephrol ..... Pediatric Nephrology

PLOS ONE.....Public Library of Science

P Natl Acad Sci USA ..... Proceedings of the National Academy of Sciences of the USA

Semin Nephrol ..... Seminars in Nephrology

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# VITA

## LEE CHUAN LI

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## EDUCATION

**Boston University**
M.S., Medical Sciences (expected 05/2015) 09/2013 – current

**University of Chicago**
B.A., Biological Sciences 09/2007 – 06/2011

## WORK EXPERIENCE

**Research Support Assistant**
University of Chicago, Chong Lab 11/2011 – 06/2013

- Investigated MRSA using ELISA, ELISPOT, and tissue culture techniques
- Analyzed data and evaluated efficacy of potential MRSA vaccines
- Designed and conducted bench and surgical experiments involving mice
- Maintained mice colonies

**Tutor and Curriculum Author**
PrepMe, Inc., Chicago, IL 10/2008 – 06/2011

- Provided personalized, one-on-one SAT/ACT tutoring for high school students
- Planned, created, and developed lesson content for online lectures
- Delivered live classroom-sized lectures online regarding standardized testing

**Stockroom Assistant**
University of Chicago 04/2010 – 06/2011

- Set up materials and reagents for laboratory teaching
- Prepared protocols for classroom assignments

## LEADERSHIP and SERVICE

**Hospital volunteering**
Boston University Medical Center 07/2014 – 08/2014
University of Chicago Medical Center 01/2012 – 03/2013

- Assisted nurses and hospital staff in patient wards
- Provided for non-medical patient comfort and care

**Physician shadowing**
University of Chicago Medical Center 01/2012 – 03/2013

- Observed procedures clinical visitation and taking patient medical history
- Performed clinical research and data analysis of lung transplantations

**Collegiate StarLeague Liason**
University of Chicago 02/2009 – 06/2011

- Coordinated online competitive video-gaming events with universities across North America
- Designed and maintained website for a Registered Student Organization
- Organized and held weekly club meetings

**Student Dormitory Council Representative**
University of Chicago 10/2008 – 06/2010

- Coordinated dormitory-wide events and activities
- Designated appropriation and use of funding

## PUBLICATIONS

Bhorade, SM, AN Husain, C Liao, LC Li, VN Ahya, MA Baz, VG Valentine, et al. 2013. "Interobserver variability in grading transbronchial lung biopsy specimens after lung transplantation." *Chest* 143 (6): 1717-1724. doi:10.1378/chest.12-2107.

## SKILLS and INTERESTS

**Computer**: proficiency in Microsoft Office, Adobe Creative Suite, HTML/CSS
**Language**: conversational fluency in Mandarin Chinese
**Personal**: music composition and piano performance